Polarized entry and release of Junín virus, a New World arenavirus

Sandra M. Cordo, Maximiliano Cesio y Acuña and Nélida A. Candurra

Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria Pab II, CP 1428, Universidad de Buenos Aires, Buenos Aires, Argentina

Junín virus (JUNV), the causative agent of Argentine haemorrhagic fever, is a human pathogen that naturally enters the body through the epithelial cells of the respiratory and digestive tracts. The interaction of JUNV with two types of polarized epithelial cultures, Vero C1008 and A549, was investigated. Radioactive virus-binding assays showed that JUNV infects polarized lines preferentially through the apical surface. High-level expression of viral nucleoprotein was detected in polarized cell lines infected through the apical domain. Virus production from apical media was about 100-fold higher than that found into the basolateral medium. Confocal-immunofluorescence analysis revealed high-level expression of glycoprotein at the apical-membrane surface. Disruption of the microtubule network by colchicine impaired JUNV vectorial release. This is the first study to analyse the interaction between a member of the virus family Arenaviridae and polarized epithelial cells, showing preferential entry and release from the apical plasma membrane.

Junín virus is a member of the family Arenaviridae and the aetiological agent of Argentine haemorrhagic fever (AHF) (Parodi et al., 1958), an endemo-epidemic disease affecting the population of the most fertile farming land of Argentina (Weissenbacher et al., 1987). Arenavirus sequence-based phylogeny comprises the Old and New World groups; JUNV is a member of the latter and belongs to the B lineage, which also includes the highly pathogenic Machupo virus, Guanarito virus and Sabiá virus (Clegg, 2002). Virions are enveloped and contain two segments of ambisense single-stranded RNA and three prominent proteins: the nucleocapsid-associated protein, NP, and two enveloped glycoproteins, GP1 (a peripheral membrane glycoprotein) and GP2 (an integral glycoprotein), derived from proteolytic cleavage of a cell-associated precursor, GPC (Romanowski, 1993).

Human infection often occurs through cuts, skin abrasions or inhalation of dust contaminated with infected rodent secretions during farming activities (Weissenbacher et al., 1987). In such cases, epithelial cells are the primary target for JUNV and are valuable in the pathogenesis of the disease.

At present, there is no information available about the interaction between any arenavirus and epithelial polarized systems; for this reason, the aim of this study was to explore the interaction between epithelial cultures and JUNV.

In previous studies, it has been demonstrated that the polarized Vero C1008 (ATCC CRL1586) and MDCK (ATCC CCL34) cell lines represent an appropriate model for the analysis of the interaction of several viruses with epithelial cells (Chen et al., 1991; Clayson et al., 1989; Srinivas et al., 1986). Similarly, A549 cells (ATCC CCL185), derived from human lung adenocarcinoma, have also been used for the study of viral pathogenesis of Human para-influenza virus 3 (Bose et al., 2001). Our initial experiments were designed to test the permissiveness of these cultures to virus multiplication and we found that, in Vero C1008 and A549 cells, the JUNV strain IV4454 (Contigiani & Sabattini, 1977) replicates as efficiently as in Vero non-polarized cells. Virus production at an m.o.i. of 0·1 in Vero C1008 cells reached titres of 10⁶ p.f.u. ml⁻¹ at 48 h post-infection, showing signs of cytopathic effect (CPE) only after 4 days p.i. Although similar titres were determined from A549-infected cultures, no CPE was evident during the first week. On the other hand, JUNV production in MDCK cells was <1×10⁶ p.f.u. ml⁻¹ at 3 days p.i. (data not shown) and, for that reason, this cell line was only used for binding and internalization assays. To determine JUNV entry, A549, MDCK, Vero C1008 and non-polarized Vero cells were grown on 0·5 µm porous-support filter inserts (Falcon). For binding experiments, a ³⁵S-methionine/cysteine-labelled JUNV preparation was purified (Damonte et al., 1994). Radiolabelled virions (1·5×10⁶ p.f.u. ml⁻¹, 2×10⁶ c.p.m. ml⁻¹) were added to either the apical or basolateral chamber of polarized and non-polarized cells and incubated at 4°C for 1 h. Then, cells were processed and associated lysate radioactivity representing the bound ³⁵S-JUNV was quantified.

We observed that the percentage of ³⁵S-JUNV bound to the apical membrane of polarized cultures represented >95%
of total binding, and cell-associated radioactivity on the basolateral domain was restricted to 3–8% in the three polarized lines (Fig. 1a). On the other hand, similar values of radiolabelled JUNV binding on both membrane domains of non-polarized cells were found. These results revealed that JUNV adsorption proceeded much more efficiently following inoculation of the apical domain of polarized cells.

Virus-internalization assays showed that the efficiency of virus uptake was independent of the inoculated surface: around 30% of adsorbed virions were internalized in both domains in polarized and non-polarized cultures (data not shown).

JUNV entry was also investigated at the level of viral-protein synthesis. A549, Vero C1008 and non-polarized Vero cells were infected by inoculation either at the apical or the basolateral surface. Cells were washed in PBS and lysed and the expression of JUNV nucleoprotein was monitored by Western blot assay using mAb NA05-AG12 from 24 to 72 h p.i. A progressive increase in the level of the NP protein band (60 kDa) in apically infected polarized cells was observed from days 1–3 p.i., whilst in the basolaterally infected cells, this protein was detected at 72 h p.i. In contrast, in non-polarized Vero cells, similar pattern of viral NP protein was seen in those cultures inoculated at the apical or basolateral surface, as expected (Fig. 1b). The slight differences in protein-synthesis levels between the apical and basolateral sides of Vero cells are likely to be due to different accessibility between the two surfaces because of the presence of the filter.

Expression of JUNV nucleoprotein was also examined by an indirect immunofluorescence assay (Candurra et al., 1999). Cultures were infected selectively through the apical or basolateral surface. Then, the intracellular presence of NP was determined by incubation with mAb IC06-BE10. Fig. 1(c) shows that apically infected Vero C1008 cells exhibit high virus permissibility. The very strong fluorescence of positive cells, forming groups of 10–20 cells, can be observed among uninfected ones. In contrast, basolaterally infected cells showed an extremely reduced and isolated number of positive cells expressing NP antigen. Each positive cell, however, exhibited the same strong staining as that seen in apically infected cells. Similar results were observed when immunofluorescent localization of NP protein was attempted on A549 cells. On the other hand, non-polarized Vero cultures showed similar NP staining, independent of the inoculation route (data not shown). Taken together, these results indicate strongly that JUNV entry is preferably localized to the apical surface of polarized cultures.

Preferential apical entry could represent the fact that the JUNV cell receptor is more abundant on this membrane domain. Our experiments suggest that, on both membrane domains, the virus uses essentially the same mechanisms of attachment and entry, which probably involve the same receptor molecules. The differences in the absolute infection rates may therefore be due to the relative concentrations of these components in the different membrane compartments of the cell. The α-dystroglycan protein has been identified as a major receptor for Old World arenaviruses and the New World C lineage (Cao et al., 1998; Spiropoulou et al., 2002). However, most New World arenaviruses, which are phylogenetically and serologically different from the former group, failed to bind this protein.
Then, taking into account the fact that \( \alpha \)-dystroglycan was found to be expressed on the basolateral membrane of epithelia in different cultured cells (Neely et al., 2001), our data suggest that the JUNV cell receptor, with preferential apical localization in polarized Vero C1008, MDCK and A549 cells, could be other than those already described for some arenaviruses.

To investigate the site of release of JUNV, cultures grown on filters were infected through the apical surface. Culture medium was collected from both the apical and basolateral chambers at different times p.i. and examined for virus production by p.f.u. assays. Polarity and integrity of the cell monolayers were monitored during the course of the infection by measuring the net transepithelial electrical resistance (TER) across the monolayer (Blau & Compans, 1995) with a Millicell-ERS apparatus (Millipore) and by optical visualization under a light microscope. Fig. 2(a) shows a graph representing JUNV release into the apical and basolateral media. The progressive increase in viral production was observed in the polarized cultures during the course of infection. Infectivity titres in apical Vero C1008 supernatants were about 100-fold higher than those determined from basolateral medium. A549 cells showed similar differences between apical and basolateral virus production. On the other hand, JUNV was released at similar levels in non-polarized Vero cells from both surface domains. For a control, we also determined the release of Vesicular stomatitis virus (VSV) in polarized Vero C1008 cells. Similar to the results observed by Fuller et al. (1984), VSV production was found predominantly in the basolateral medium (data not shown). Additionally, virus production in polarized cells was also evaluated with several JUNV strains, including the prototype XJ and the host-range mutant XJC167 (Scolaro et al., 1989), as well as the closely related New World Tacaribe virus (TCRV). Vero C1008 cells were infected by inoculation at the apical membrane and virus yield was determined at 48 h p.i. The infectivity values corresponding to the apical chamber were about 100-fold higher than those found in the basolateral chamber (Fig. 2b). These results indicated that the preferential release of JUNV strains from the apical side of polarized cells was shared by TCRV.

Most viral glycoproteins are transported to the membrane compartment, where release of the virus takes place. To investigate JUNV-glycoprotein transport, we analysed the surface distribution of GP1 on infected cells with mAb GB03-BE08 by confocal microscopy. Cultures were grown on filters, infected through the apical surface and processed for membrane immunofluorescence. Fig. 2(c) shows a z-section image of an infected Vero C1008 cell monolayer after 48 h p.i. The immunofluorescence staining of these cells was restricted to the apical surface. Thus, we concluded that the surface expression of JUNV glycoproteins is polar and that this protein is expressed on the same surface from which the virus is released in polarized Vero C1008 cells.

The microtubule network has been reported to participate in vectorial targeting of sphingolipids (Zegers et al., 1998).

**Fig. 2.** Preferential apical release of JUNV from polarized cells. (a) Cultures were grown on filters and infected through the apical surface. The supernatant medium was collected at different times p.i. from both the apical (shaded bars) and basolateral (empty bars) chambers. The released-virus titres were examined by p.f.u. assays. (b) JUNV and TCRV release from Vero C1008 cells. Polarized cultures were infected with three different strains of JUNV and with TCRV through the apical surface. The supernatants from both the apical (shaded bars) and basolateral (empty bars) chambers were collected at 48 h p.i. and virus release was examined by p.f.u. assays. Each value is the mean ± SD of duplicate independent experiments. (c) Apical localization of the JUNV glycoproteins in polarized Vero C1008 cells by confocal microscopy. Cell monolayers were grown on filters and infected with JUNV through the apical membrane. After 48 h p.i., filters, with attached cells, were removed and cell-surface staining of JUNV glycoproteins was examined by indirect immunofluorescence. The z section of the infected cells (bottom panel) shows apical immunofluorescence.
and membrane-protein targeting (Jacob et al., 2003; Lafont et al., 1994) and secretion (Guadiz et al., 1997) to the apical surface in epithelial cells. Also, the importance of the microtubules and motor proteins in the polarized budding and egress of several viruses has been reported (Bose et al., 2001; Chu & Ng, 2002; Marozin et al., 2004).

In order to examine the role of the cytoskeleton in the polarized release of JUNV, immunofluorescence studies were performed with drug treatments affecting microfilaments, microtubules and intermediate filaments. The effect of anticytoskeletal drugs on cell viability and TER was also tested, to assure that the monolayers were still effective permeability barriers (data not shown). Polarized cells grown on filter inserts were infected apically, depolymerizing drugs were added to the culture medium and infectivity on both domains was determined by p.f.u. assays at 24 h p.i. According to previous results (Candurra et al., 1999; Cordo & Candurra, 2003), a decrease in overall JUNV production was seen in Vero C1008 cell cultures in the presence of anticytoskeletal drugs. However, only disruption of the microtubule network led to a non-polarized release of JUNV infectivity (Fig. 3). Similar consequences were seen in infected A549 cells after equal treatment periods (data not shown). The requirement of microtubule integrity for polarized JUNV production would suggest the involvement of this network in both appropriate vectorial transport and membrane address of JUNV proteins.

Viruses that show polarized entry and release from epithelial cells at the apical membrane may be restricted to these epithelial surfaces and appear to produce a localized infection, such as those produced by Sendai virus and Simian virus 5 (Schneider-Schaulies, 2000). In contrast, Measles virus (MeV) produces a systemic infection in vivo and its entry and release take place predominantly at the apical surface. MeV can infect cells of the immune system; thus, cells may traverse the epithelial barrier and could introduce the virus to underlying tissues or to the bloodstream (Blauf & Comans, 1995). On the other hand, apical release of coronavirus into the lumen of the gut was suggested to allow rapid infection of adjacent cells in the epithelium by lateral dissemination, improving virus production (Rossen et al., 1994).

It has been shown that macrophages and splenic dendritic cells are the first targets associated in vivo with JUNV (Gonzalez et al., 1980; Laguens et al., 1983). Thus, immune-system cells could be implicated in JUNV spreading and pathogenesis. Also, apical release from the epithelial surface could be advantageous for JUNV progeny, which also penetrate the cells from the apical side. The other, non-exclusive possibility is that, although JUNV showed preferential apical egress in epithelial cultures, the proportion of viruses released at the basal membrane may be sufficient for the dissemination and development of systemic infection. Future characterization of molecular determinants of JUNV apical targeting in epithelial cultures will improve the understanding of AHF pathogenesis.

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References


