In the present work we investigated the importance of the Raf/MEK/ERK signalling pathway in the multiplication of the arenavirus Junin (JUNV) in monkey and human cell cultures. We established that JUNV induces a biphasic activation of ERK and we proved that a specific inhibitor of the ERK pathway, U0126, impairs viral replication. Furthermore, U0126 exerted inhibitory action against the arenaviruses Tacaribe and Pichinde. Moreover, treatment with known ERK activators such as phorbol 12-myristate 13-acetate and serum increased viral yields whereas ERK silencing by small interfering RNAs caused the inhibition of viral multiplication. Therefore, activation of the Raf/MEK/ERK signalling pathway is required to ensure efficient JUNV replication and may constitute a host target for the development of novel effective therapeutic strategies to deal with arenavirus infections.

Arenaviruses are enveloped viruses containing a bisegmented single-stranded RNA genome with ambisense coding strategy. This family includes important emerging pathogens such as lymphocytic choriomeningitis virus (LCMV), implicated in post-transplant fatalities and congenital infections, and several haemorrhagic-fever-causing viruses, such as Lassa (LASV), Junin (JUNV), Sabia, Guanarito, Chapare and Machupo viruses (Emonet et al., 2009). JUNV is the aetiological agent of Argentine haemorrhagic fever (AHF) and the administration of defined doses of convalescent plasma is until now the best therapy against AHF. Although different types of compounds exhibit antiviral activity against arenaviruses (Garcia et al., 2011), no specific and safe chemotheraphy for these viruses is currently available (Gomez et al., 2011; McLay et al., 2013).

Virus infections modulate a variety of intracellular signalling pathways to ensure efficient replication. The Raf/MEK/ERK signal transduction pathway is one of the mitogen-activated protein kinase (MAPK) cascades involved in signal transduction from the cell membrane to the nucleus. The Raf/MEK/ERK pathway comprises a set of three sequentially acting kinases: the serine threonine kinase Raf, the kinase MEK and the extracellular-signal-regulated kinases 1 and 2 (ERK 1/2). Active ERK 1/2 in turn phosphorylate a variety of substrates leading to changes in gene expression, cell metabolism or apoptosis induction. Many DNA viruses induce the Raf/MEK/ERK pathway leading cells into a proliferative state; however, infection with several human pathogenic RNA viruses (Pleschka, 2008), including influenza viruses (Ludwig, 2009), picornaviruses (Luo et al., 2002; Wong et al., 2005), Borna disease virus (BDV) (Planz et al., 2001), Ebola virus (Zampieri et al., 2007), Measles virus (MV) (Torres et al., 2012b) and flaviviruses (Huerta-Zepeda et al., 2008; Grettom et al., 2010; Scherbik & Brinton, 2010; Yang et al., 2010; Zhao et al., 2013), also activate ERK 1/2.

Specific inhibitors, which block activation of MEK, have been used to elucidate the function of this kinase cascade in cellular regulation, with U0126 being one of the most frequently employed. U0126 inhibitory action is specific with little, if any, effect on other protein kinases (Planz, 2013). Blockade of ERK phosphorylation impairs growth of influenzavirus A and B (Droebner et al., 2011; Pinto et al., 2011), coxsackievirus B3 (CV B3) (Luo et al., 2002), enterovirus 71 (Wang et al., 2013), Hepatitis C virus (Grettom et al., 2010), MV (Torres et al., 2012b) and BDV (Planz et al., 2001).

The roles of signalling pathways that control basic cellular metabolism and homeostasis in arenavirus replication are still poorly understood. Since many of these viruses have tropism for mononuclear phagocytes, which possess a central role in mediating immune responses, alteration of basic functions may contribute to viral pathogenesis (Bowick & McAuley, 2011). A kinomic study revealed a differential phosphorylation status of ERK in guinea pig macrophages infected with a virulent or an attenuated strain of the arenavirus Pichinde (PICV) (Bowick et al., 2007). Furthermore, infection of Vero cells with PICV induces ERK phosphorylation (Vela et al., 2008) and LCMV infection also causes ERK phosphorylation in
CD1d1-expressing cells (Renukaradhya et al., 2005); on the contrary, LASV infection impairs MEK/ERK activation after virus binding to its receptor (Rojek et al., 2012).

Here we investigated the importance of the Raf/MEK/ERK signalling pathway in JUNV multiplication in monkey and human cell cultures. We first investigated if JUNV infection induces ERK activation in Vero (monkey kidney fibroblasts, ATCC CCL-81), A549 (human epithelial lung cells, ATCC CCL-185) and U937 (human monocytes, ATCC CRL-2367) cell lines. Cells were infected with JUNV (strain XJCl3), incubated in Eagle’s minimum essential medium (MEM; Gibco) without serum and at 24 h post-infection (p.i.) they were lysed and ERK phosphorylation was determined by Western blot (WB) using rabbit anti-ERK 1 or anti-p-ERK 1/2 antibodies (sc 94 and sc16982; Santa Cruz Biotechnology) (Torres et al., 2012a). In the three cell systems analysed, JUNV infection increased ERK phosphorylation in comparison to the basal levels of p-ERK in uninfected cells (Fig. 1a). Next, we examined ERK phosphorylation at different times after JUNV infection in Vero cells. We observed a biphasic activation of ERK: an early activation within the first 30 min of infection, followed by a decrease in ERK phosphorylation to basal levels and a second phase of activation from 7 h p.i. onwards (Fig. 1b). We did not detect variations in p-ERK levels in mock-infected cells used as control (Fig. 1b, mock). To understand the significance of ERK activation for viral replication we examined the effect of U0126 (Promega) on ERK phosphorylation in Vero cells infected with JUNV or with the arenaviruses PICV and Tacaribe (TCRV). After 1 h of adsorption, cells were covered with MEM without serum containing different concentrations of U0126 for 24 h. The lack of cytotoxic effect of U0126 treatments was determined as previously described (Torres et al., 2012a) (data not shown). U0126 inhibited ERK phosphorylation in a dose-dependent manner (Fig. 2a) and virus yields, assessed by a plaque assay, were reduced by more than 99% by 20 μM U0126 (Fig. 2b). The number of cells expressing JUNV nucleoprotein N, detected by an immunofluorescence assay using anti-N protein mAb SA02 BG12 and FITC-conjugated goat anti-mouse IgG (Maeto et al., 2011; Sánchez et al., 1989), was also reduced at concentrations of 5 μM and 20 μM U0126 compared to the control (Fig. 2c). In addition, JUNV replication and ERK phosphorylation in A549 and U937 cells were also affected by U0126 treatment.

![Fig. 1. JUNV infection induces ERK phosphorylation. Mock-infected or JUNV-infected (m.o.i. =1) Vero, A549 or U937 cells were lysed at 24 h p.i. and ERK activation was analysed based on the levels of phosphorylated ERK (p-ERK) and total ERK determined by WB (a). JUNV-infected (m.o.i. =1) or mock-infected Vero cells were lysed at different times after infection and levels of p-ERK and ERK were assessed by WB (b). Each value represents the mean ± SD of three independent experiments. Statistical significance of the differences between JUNV-infected and mock-infected cells was calculated using the Student’s t-test (*P<0.05).](image-url)
Fig. 2. U0126 inhibits JUNV multiplication. Vero cells were infected with JUNV, PICV or TCRV (m.o.i. = 1) and after 1 h of adsorption different concentrations of U0126 were added. After 24 h, p-ERK and ERK levels were assessed by WB (a). JUNV, PICV or TCRV-infected cells were treated with U0126 (20 μM) for 24 h and extracellular virus production was measured by plaque assay (b). N protein expression detected by an immunofluorescence assay in JUNV-infected cells treated with different concentrations of U0126 for 24 h. The percentage of cells expressing N protein was calculated by counting 20 randomly selected fields of each sample (c). A549 or U937 cells were infected with JUNV (m.o.i. = 1) and after 1 h of adsorption different concentrations of U0126 were added. After 24 h, virus yields were quantified by plaque assay (d). Virus yields were determined by plaque assay in supernatants obtained at 24 h p.i. from JUNV-infected Vero cells untreated (virus control: VC) or treated with U0126 (20 μM) from 1 h prior to infection (−1 h) or from 1 or 5 h p.i. (e). Data are mean ± SD from triplicate experiments. Statistical significance of the differences between U0126 treated cells and untreated cells was calculated using the Student’s t-test (*P < 0.05).
as can be observed in Fig. 2(d) and Fig. S1(a) (available in the online Supplementary Material), respectively.

Since, in accordance with results obtained with other viruses such as *Influenza A virus*, CV B3 and herpes simplex virus type 1 (Luo et al., 2002; Pleschka et al., 2001; Torres et al., 2012a), an early and late phase of ERK activation was detected (Fig. 1b), we investigated whether both activation events are required for virus multiplication. To this end, we analysed the effect of 20 μM U0126 addition either 1 h prior to infection (−1h) or at 1 h or 5 h p.i. In all cases U0126 was present up to 24 h p.i. when virus titres were determined (Fig. 2e). Maximum inhibition of virus yield was obtained when U0126 was added 1 h prior to infection, whereas a reduction of 2.5 logarithm units in virus titres was still detected in cells treated from either 1 h or 5 h p.i., suggesting that both activation events contribute to viral infectivity. Coincidently, the highest inhibition of the expression of viral protein N and the precursor of JUNV glycoproteins called GPC, detected by WB using NA05AG12 and QD04-AF03 mAbs as primary antibodies (Sánchez et al., 1989), was observed when the compound was added at 1 h before infection (Fig. S1b). These results strongly indicate that the initial phase of ERK activation, probably triggered by virus entry, is critically relevant for an efficient infection. Nevertheless, the inhibition of virus production achieved by U0126 when added at 5 h p.i. would indicate that the compound may also inhibit a late stage of the viral cycle as has been described for influenza virus A (Pleschka et al., 2001).

To further corroborate the involvement of the Raf/MEK/ERK pathway in JUNV multiplication we investigated the effect of ERK silencing on viral production. To this end, we transfected Vero and A549 cells with a pool of six small interfering RNAs (siRNAs) against ERK1/2 (Steinmetz et al., 2004) using Lipofectamine 2000 (Invitrogen). The sequences of the siRNAs used were: 5′-GACCAGGAUG-UUAACCUUUAA3′, 5′-GAAACUACGUAGUUGCAUA-3′, 5′-GCUAGCGGAGUGGAUAUUA-3′ and 5′-GUACAGGGC-UCCAGAAAUU-3′ to inhibit ERK1 expression and 5′-CCAAAGCUCUGACCUUUAAU-3′, 5′-CAAGGAAUUGAAGAAGA-3′ and 5′-GUACAGGGC-UCCAGAAAUU-3′ to inhibit ERK2 expression. The sequence of the RNA used as control was 5′-GACCGAAUUGCCAGTATACAUU-3′. Cells were transfected with 300 nM siRNA or control RNA and after 24 h a second round of transfection was performed. At 24 h post-transfection cells were infected with JUNV and virus production was quantified at 24 h p.i. Fig. 3(a) shows that siRNA transfection rendered a marked inhibition of ERK expression analysed at 24 h post-transfection when virus infection was performed. The lack of toxic effect of RNA transfection was assessed by the trypan blue exclusion method (data not shown). ERK silencing caused 65.5% and 91.6% reduction in JUNV yields in Vero and A549 cells, respectively (Fig. 3b). Fig. 3(c) shows the inhibitory effect of siRNA transfection on the expression of N protein in A549 cells, corroborating the involvement of ERK on JUNV replication.

Diverse extracellular stimuli are known to activate ERK, including serum growth factors, thrombin, and phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (Abkhez et al., 2010; Verin et al., 2000). PMA modulates diverse cellular responses through the activation of protein kinase C (PKC) and one well-studied mode of PKC-mediated signalling involves activation of the Raf/MEK/ERK pathway (Chang et al., 2005). To examine the effect of PMA and serum on JUNV multiplication, Vero cells infected with JUNV were incubated at 1 h p.i. with MEM containing different concentrations of PMA or 20% newborn calf serum (NBCS, GIBCO) for 24 h. A 6.5-fold and 12-fold increase in virus titre was obtained with 100 nM PMA (Fig. 3d) and 20% NBCS (Fig. 3e), respectively. An augmentation in ERK phosphorylation was observed in mock- and JUNV-infected cells treated with increasing concentrations of PMA (Fig. 3f) that correlates with the increase of virus yields in these cultures (Fig. 3d). Serum is able to activate several cell signalling cascades including the PI3K-Akt pathway (Abkhez et al., 2010) and the latter would be required for JUNV entry into the cell (Linero & Scolaro, 2009). In order to rule out serum effects unrelated to ERK activation we investigated whether NBCS could counteract U0126 inhibitory action. To this end, JUNV-infected cells were incubated with MEM containing U0126 (14 μM) and different concentrations of NBCS. The presence of serum re-established viral infectivity (Fig. S2a) and partially restored ERK phosphorylation (Fig. S2b), supporting the idea that NBCS promotes viral multiplication by the activation of the Raf/MEK/ERK pathway.

In conclusion, here we demonstrate that JUNV infection induces ERK phosphorylation, which is critical for virus replication. Specific inhibition of the Raf/MEK/ERK pathway strongly reduces JUNV multiplication and also inhibits the replication of two other arenaviruses, PICV and TCVR. Reinforcing the role of the MAPK cascade in JUNV infectivity, compounds that stimulate the ERK pathway, such as serum or PMA, favour virus replication.

The molecular mechanism underlying JUNV activation of ERK signalling requires further investigation. The results obtained so far do not allow us to distinguish whether ERK activation leads to a direct modification of viral proteins or to an indirect effect produced by an altered cell state. It has been reported that arenavirus N protein would be phosphorylated via serine and threonine residues (Howard & Buchmeier, 1983), so one possibility is that this viral protein could play a role in JUNV interactions with cell signalling pathways.

PMA induces ERK phosphorylation in endothelial cells, leading to disruption of monolayer integrity and intercellular gap formation (Verin et al., 2000). Compromise of endothelial cell barrier integrity leads to an increase in vascular permeability; therefore, JUNV activation of the ERK pathway might be a main factor involved in viral pathogenesis in vivo. Blockade of signalling pathways
involved in immunopathology and cellular deregulation during haemorrhagic fevers may be an effective therapeutic strategy with a low risk of emergence of viral resistance, being also effective against several viruses that exploit similar cellular pathways (Linero et al., 2012). However, targeting host factors might result in undesirable side-effects, hence host-based antiviral strategies seem to be suitable for pathogens associated with acute disease in humans, like haemorrhagic arenaviruses, which may be controlled by short-term treatments.

**Fig. 3.** Silencing or activation of ERK modulates JUNV multiplication. Vero or A549 cells were transfected with ERK siRNAs (siRNA) or control RNA (cRNA), and at 24 h post-transfection the level of ERK expression was assessed by WB (a). Another set of identically transfected cultures were infected at 24 h post-transfection with JUNV (m.o.i. = 1) and at 24 h p.i. viral yield was measured by plaque assay (b) and N-protein expression was analysed by immunofluorescence (c). Vero cells were infected with JUNV (m.o.i. = 1) and after adsorption MEM containing different concentrations of PMA (d) or 20% NBCS (e) was added and 24 h later virus yields were determined by plaque assay. Mock-infected or JUNV-infected Vero cells treated with different concentrations of PMA for 24 h were lysed and p-ERK and ERK were detected by WB (f). In (b), (d), (e) and (f) data are mean ± SD from triplicate experiments. Statistical significance of the differences between treated and control cultures was calculated using the Student’s t-test (*P < 0.05).
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References


