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Short communication

Participation of the phosphatidylinositol 3-kinase/Akt pathway in Junín virus replication in vitro

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ABSTRACT

In this paper we demonstrate that infection of cell cultures with the arenavirus Junín (JUNV), agent of the argentine haemorrhagic fever, leads to the activation of PI3K/Akt signalling pathway. Phosphorylation of Akt occurs early during JUNV infection of Vero cells and is blocked by the PI3K inhibitor, Ly294002. Infection of cells with UV-irradiated JUNV redeemed the pattern of stimulation observed for infectious virus indicating that an early stage of multiplication cycle would be enough to trigger activation. Treatment of cells with chlorpromazine abrogated phosphorylation of Akt upon JUNV infection suggesting virus internalization as responsible for activation. Inhibition of Akt phosphorylation by Ly294002 impaired viral protein synthesis and expression leading to a reduced infectious virus yield without blocking the onset of persistent stage of infection. This impairment is linked to a reduced amount of virus bound to cells probably due to a blockage on the recycling of transferrin cell-receptor, employed by the virus to adsorb to the cell surface. Early Akt activation was also observed in BHK-21 and A549 JUNV infected cells suggesting an important role of PI3K/Akt signalling in JUNV multiplication in vitro.

Junín virus (JUNV), agent of the Argentine haemorrhagic fever, belongs to the *Arenaviridae* family, a group of enveloped viruses with genome composed of two negative-sense single stranded RNA segments (L and S) that encode genes of viral proteins, N, L, GPC (G1 and G2) and Z, using an ambisense coding strategy (Buchmeier, 2002; Meyer et al., 2002). Previous findings suggest that arenavirus pathogenesis could well involve the dysregulation of cytokines and immune signalling controlled by the initial response to activation of a series of phosphorylation events (Bowick et al., 2007). However, it is not well understood how arenaviruses induce cell-signalling changes, which lead to this dysregulation and potentially to clinical disease. Many viruses have evolved mechanisms to gain control of key cellular signalling pathways that affect broad aspects of cellular macromolecular synthesis, metabolism, growth and survival. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) is one of such pathways promoting cell survival by phosphorylation and inhibition of a number of pro-apoptotic proteins. Both DNA and RNA viruses must regulate this pathway, either by activating or inactivating some aspect of it, in order to achieve an efficient replication process (Cooray, 2004; Buchkovich et al., 2008).

In this study we examined the role of PI3K/Akt signalling pathway during JUNV in vitro infection. In first place, we tested whether JUNV infection would lead to activation of PI3K/Akt pathway by examining the kinetics of Akt phosphorylation. To this end, Vero 181 cells were infected with JUNV strain XJCl3 and further incubated at 37 °C for 15, 30 and 60 min when cultures were processed for Western blotting (WB). For later samples (90, 120 and 240 min p.i.), virus was removed after 60 min of virus-cell contact and serum free medium was added to the cultures. Time zero post-infection (p.i.) corresponds to the inoculation time point. Proteins from whole-cell lysates were separated by 10% SDS-PAGE, transferred onto a PVDF membrane, as previously described (Ellenberg et al., 2004) and revealed by using an anti-phospho-Akt antibody. Membranes were stripped and re-probed with anti-Akt total antibody to assure equal protein loading. As can be seen in Fig. 1a, infection with JUNV induced early Akt activation, peaking at 15–30 min p.i., sustaining thereafter a moderate level of phosphorylation up to 120 min p.i. which decreased at 240 min p.i., when the level of phosphorylated Akt of infected cells showed a faint band slightly darker than mock infected control (Fig. 1a and b). A similar result was obtained in JUNV infected BHK-21 cells (Fig. 1c).

Taken into consideration that Akt activation was an early event during infection, we speculated this activation might be due to the initial virus–host cell interactions. To test this hypothesis we inoculated Vero cells, under the same conditions described above, with UV-irradiated JUNV, prepared by exposing infectious virus suspended in PBS to a 30 W UV light at a distance of 10 cm for 3 min. Inactivation of virus was inferred from the fact that Vero cells infected with UV-inactivated JUNV were not able to express

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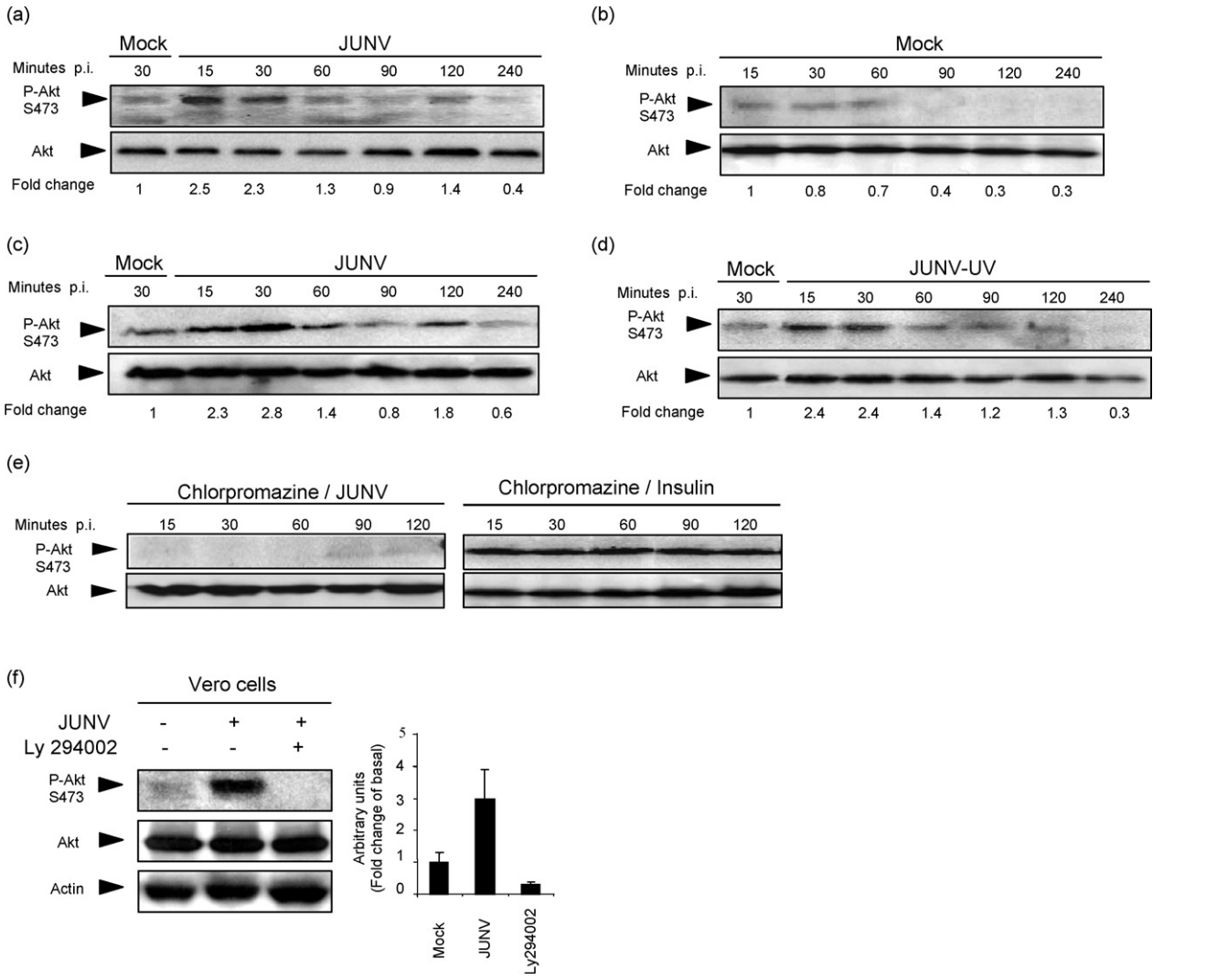

Fig. 1. JUNV infection induces Akt phosphorylation via a PI3K-dependent pathway. Vero (a) and BHK-21 (c) cells were infected with XJCl3 strain of JUNV at an MOI of 1 PFU/cell and processed for WB at the indicated times p.i. Mock infected Vero (b and first lines in a and d) and BHK-21 (first line in c) cells and Vero cells infected with UV-inactivated JUNV (d) were used as controls. Vero cells pre-treated for 2 h with 40 μM chlorpromazine were infected with JUNV at an MOI of 1 PFU/cell (e, left panel) or incubated with 240 μM insulin (e, right panel) and processed for WB at indicated times p.i. Vero cells treated with 10 μM Ly294002 (f) were infected with JUNV at an MOI of 1 PFU/cell and processed for WB at 30 min p.i. Phosphorylation of Akt (Ser473) was determined by WB in whole-cell lysates using a phospho-Akt (Ser473) (CST 9271, New England Biolabs) or total Akt (CST 9272, New England Biolabs) antibodies. Fold change of Akt phosphorylation was expressed as densitometric units (Scion Image software) of band normalized to the total Akt level relative to the uninfected control from of each panel. All experiments were performed on 24 h serum starved cells, and the infection and treatments were carried out in serum free medium.

viral proteins detected by WB and failed to yield infectious virus quantified by plaque assay (data not shown). As can be seen in Fig. 1d, UV-irradiated JUNV also led to an early Akt phosphorylation event occurring at 15–30 min p.i. at a level of stimulation comparable to that obtained with control virus (Fig. 1a) suggesting that an early step of JUNV multiplication would be responsible for Akt activation. Activation of Akt by initial virus–host cell interactions has been also described for HIV (Briand et al., 1997) and enterovirus 71 (Wong et al., 2005). This was consistent with the fact that when we analyzed the activation of Akt by transferrin (Tf), a ligand for the receptor employed by JUNV to enter into many cell types (Flanagan et al., 2008; Radoshitzky et al., 2007), a similar kinetics of Akt activation was observed (data not shown). Moreover, the pattern of Akt activation induced by JUNV and Tf was also reported for the interaction between the insulin-like growth factor type I receptor (IGF-IR) and its ligand. Previous reports showed that this receptor co-localizes with Tf receptor in endosomes and recycles in a similar fashion depending on and sustaining, at the same time, Akt phosphorylation (Van Dam et al., 2002; Romanelli et al., 2007).

The fact that the level of phosporylation of Akt in JUNV infected cells analyzed at later times p.i. (6, 12 and 18 h p.i.) did not differ from non-infected cultures prompted us to conclude that activation of this pathway was not dependant on JUNV protein synthesis (data not shown).

Taken into consideration that endocytic uptake is also an early event in virus cycle and that activation of PI3K/Akt pathway has been linked to the entry of Ebola virus (Saeed et al., 2008) and rhinovirus (Lau et al., 2008), we investigated whether this mechanism might be responsible for Akt activation by JUNV, which enters cells employing a receptor-mediated endocytosis mechanism involving clathrin participation (Martinez et al., 2007). To this end, we tested the effect of chlorpromazine, an inhibitor of clathrin-mediated endocythosis, on Akt phosphorylation induced by JUNV infection.

As can be seen in Fig. 1e, treatment of Vero cells with 40 μM chlorpromazine abolished Akt phosphorylation induced by JUNV infection, strongly suggesting that entry of virus into cells is responsible for the observed activation. To discard a direct effect of the drug on Akt phosphorylation, insulin, a ligand that activates Akt by

direct binding to its receptor, was used as a control. Treatment of cells with chlorpromazine did not impair Akt activation induced by insulin, indicating that insulin treated cells were able to activate the pathway in the presence of this drug (Fig. 1e). The effect observed for chlorpromazine treated JUNV infected cells is in accordance with the blockage of Akt phosphorylation observed when the internalization of IGF-IR was impaired by treatment of cells with dansylcadaverine, an inhibitor of clathrin-mediated endocythosis (Romanelli et al., 2007).

In view that Akt constitutes a major downstream effector of PI3K, we next analyzed phosphorylation of Akt induced by JUNV in the presence of Ly294002, a specific inhibitor of PI3K. On this purpose, Vero cells infected with JUNV in the presence of 10 μM Ly294002 were processed for WB at 30 min p.i. to determine phosphorylated Akt. As can be seen in Fig. 1f, treatment of cells with Ly294002 inhibited JUNV induced phosphorylation of Akt without affecting the level of expression of total Akt, suggesting that this activation is mediated by PI3K. We also determined whether this effect was a cell-specific phenomenon. On this purpose, Akt activation and Ly294002 effect on virus multiplication was analyzed in BHK-21 and A549 cells. JUNV infection of both cell types led to an early Akt activation in a similar manner described for Vero cells, peaking in both cases at 15–30 min p.i. (data not shown).

To investigate whether the activation of Akt by JUNV plays a role in virus replication, we examined the effect of the specific PI3K inhibitor, Ly294002 on virus production in Vero and BHK-21 cells. As can be seen in Fig. 2a, virus infectivity recovered from JUNV-infected cells treated with Ly294002 was reduced in comparison with control samples. In fact, both, Vero and BHK-21 infected cultures treated with the compound showed a five-fold decrease in virus titer (Fig. 2a). Cell viability for both cell types was similar to untreated cultures (data not shown). Consistent results were obtained when the effect of PI3K/Akt inhibition by Ly294002 on viral protein synthesis and expression was analyzed by WB and indirect immunofluorescence assay (IFA), respectively. JUNV infected cells treated or not with the inhibitor were collected at 24 h p.i., subjected to WB and revealed with an anti-N Mab NA05-AG12 (Sanchez et al., 1989). Alternatively, cells grown in coverslips treated under the same conditions mentioned above, were fixed with methanol during 15 min at −20 °C and processed for IFA, as previously described (Ellenberg et al., 2004), employing anti-N Mab SA02-BG12 (Sanchez et al., 1989). As shown in Fig. 2b, treatment of infected cells with Ly294002 led to an inhibition of N expression, detected by IFA, reaching 85 and 87% of inhibition for Vero and BHK-21 cells, respectively. To note, although N fluorescence pattern in treated cells remained unchanged, proportion and fluorescence intensity of positive cells was markedly reduced compared to untreated control cells. Similarly, as can be seen in Fig. 2c, inhibition of N synthesis, detected by WB, was in accordance with the results mentioned above. Also, the presence of Ly294002 in A549 JUNV infected cells reduced the synthesis and expression of N, suggesting that this phenomenon is not dependent on cell origin (data not shown). These results, indicating the participation of PI3K/Akt pathway in JUNV multiplication, were confirmed when a dominant-negative mutant for Akt was employed (Murga et al., 1998). As can be seen in Fig. 2d, JUNV infection of Vero cells that had been previously transfected with a pCEFL vector encoding wild-type Akt (PKB-wt) showed a higher proportion of cells bearing both green (Akt) and red (N) fluorescence (62 ± 5%) in comparison with infected cells transfected with a kinase-inactive mutant of Akt (PKB-kd) which showed a reduced percentage of cells bearing both fluorescent marks (13 ± 8%) ($p < 0.01$), suggesting that dominant-negative mutant of Akt impaired N synthesis. This situation also leaded to a reduced viral yield of infected cells that had been previously transfected with the dominant-negative mutant (Fig. 2e). On the contrary, vesicular stomatitis virus multiplication was not affected by the dominant-negative mutant in accordance with previous results demonstrating that this virus is not affected by Ly294002 (Saeed et al., 2008).

Addition of Ly294002 at different times p.i. during JUNV multiplication cycle showed that the inhibition was exerted during early times of infection, losing its effect when added after 3 h p.i. (Fig. 2f) indicating that requirement for PI3K activity in JUNV multiplication cycle is restricted to the early stages of infection. This was confirmed when the initial steps of the multiplication cycle were studied in a combined assay. To this end, Vero cells were infected with JUNV in the presence or not of Ly294002, and incubated at 37 °C in order to allow adsorption, penetration and uncoating of virions. At different incubation times cells were washed with cold PBS, lysed by freeze-thawing and infectivity quantified by plaque assay. As can be seen in Fig. 2g a maximum value of internalized infectivity was observed at 30 min post-contact for treated and untreated cells as well, followed by a decrease in a time course dependant manner in both cases. This result indicates that uncoating occurred in control and treated cells at a similar rate, however, the amount of cell-associated virus was significantly lower in the case of cells treated with Ly294002 suggesting an impairment in the uptake of virus to treated cells. As mentioned above and considering that JUNV uses transferrin receptor to adsorb to cells, blockage of recycling of transferrin receptor caused by Ly294002 might account for the diminished levels of cell associated virus. In fact, non-infected Vero cells incorporated labelled transferrin that accumulated after 30 min of contact around the nucleous defining an intense perinuclear region whereas Ly294002 treated cultures showed a low intensity punctuate pattern scattered in the cytoplasm (Fig. 2h). These results allow us to speculate that impairment of JUNV adsorption to Vero cells treated with Ly294002 might be due a blockage in transferrin receptor recycling. To test this hypothesis, Vero cells treated with Ly294002 were incubated with transferrin (in order to obtain cell surfaces depleted of transferrin receptors), and further infected with JUNV. As can be seen in Fig. 2i, a significant reduction in virus titer was observed in Ly294002 treated cells that had been incubated with transferrin suggesting that recycling of transferrin receptor is necessary for an optimal virus binding to cells. Treatment of cultures with Ly294002 per se did not affect binding of JUNV to Vero cells (Fig. 2i). This finding may be explained by the fact that when an infection is carried out at a low MOI it would be enough the presence of one cell-receptor to allow virus adsorption.

In this paper we demonstrate that JUNV infection of cell cultures is able to activate PI3K/Akt signalling pathway at an early stage of infection. This activation, mediated by virus internalization, would be necessary for an efficient viral multiplication, particularly, virus adsorption to cells mediated by transferrin receptor. A direct effect on Akt phosporylation inhibition, mediated by Ly294002, on GTPase Rab5, a known regulator of early endosome fusion (Li et al., 1995), cannot be discarded, however the observation that JUNV is able to uncoat in Ly294002 treated cells does not support this idea.

In view that it has been reported for SARS-CoV, that activation of PI3K was necessary not only for virus multiplication during acute infection but for the establishment of persistence as well (Mizutani et al., 2005), and considering that JUNV is able to establish persistent infections in the majority of cell lines it infects, we decided to analyze the effect of Ly294002 on the establishment of this type of infection. We observed that impairment of viral multiplication by Ly294002 did not affect establishment of persistence. The reduced proportion of infected cells that remained after a two weeks Ly294002 treatment, led to the establishment of persistently infected cultures characterized by a continuous synthesis of N and reduced levels of infectivity, suggesting the drug was not able to modify the transition from acute to persistent infection (data not shown). It remains to be elucidated the role of Akt in the maintenance of this stage of infection, particularly focused on control of

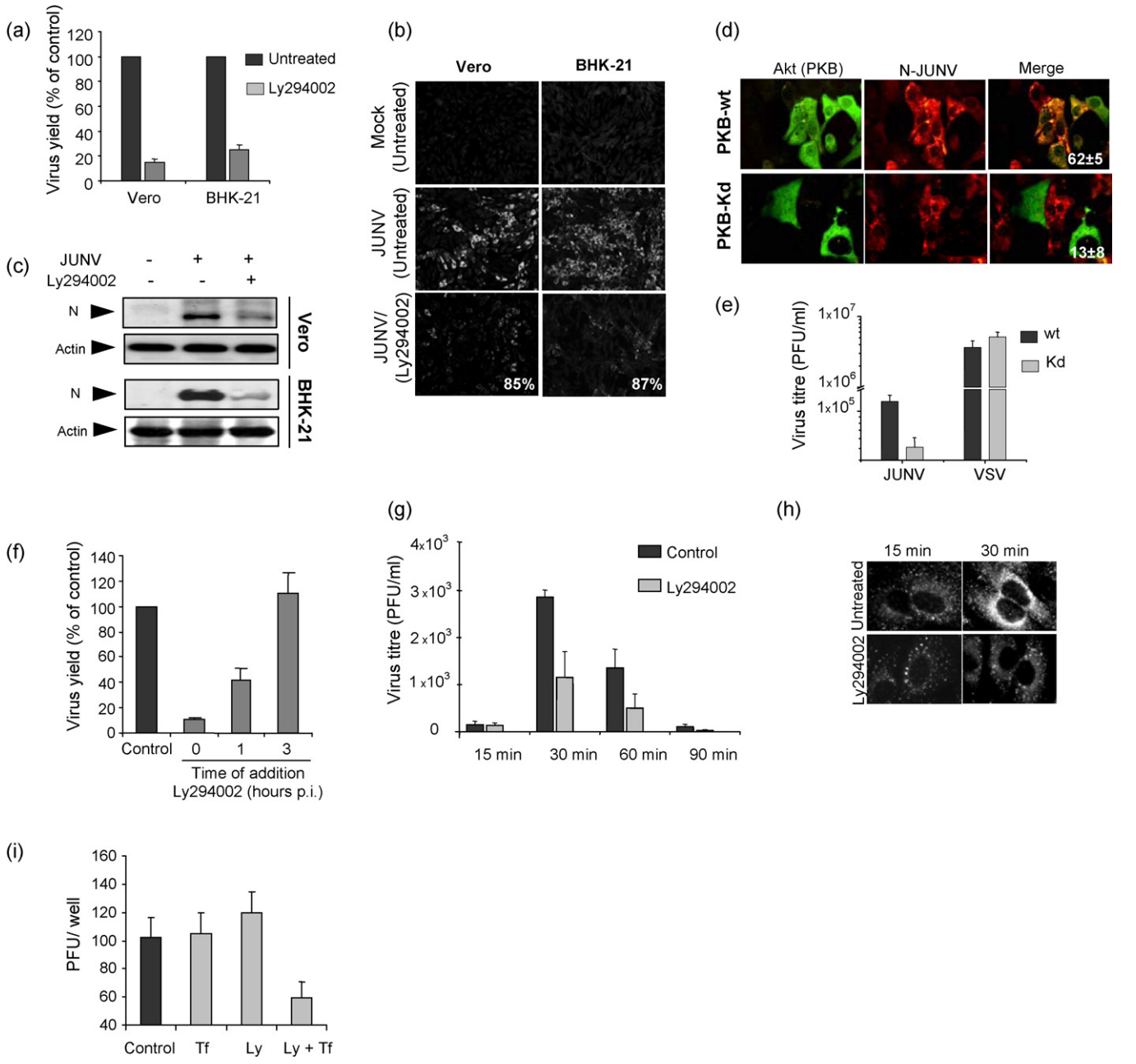

Fig. 2. Inactivation of PI3K/Akt pathway impairs JUNV multiplication. (a) Vero and BHK-21 cells were infected with JUNV at an MOI of 1 PFU/cell and treated with 10 μM Ly294002 (Sigma, MO). At 24 h p.i. supernatants were collected and virus titer was calculated by plaque assay. Alternatively, cells treated under the same conditions mentioned above were fixed with methanol and subjected to IFA (b) or processed for WB (c) to determine N synthesis and expression. Values in b) indicate % of inhibition calculated from amounts of fluorescence intensities (ImagePro-Express software) in treated cells in comparison to untreated controls. Similar numbers of total cells were analyzed for each category. Vero cells transfected either with pCEFL vector encoding wild-type Akt (PKB-wt) or a kinase-inactive mutant (PKB-kd) were infected with JUNV at an MOI of 10 PFU/cell at 24 h post-transfection and fixed at 24 h p.i. with methanol for IFA, employing anti-N (red fluorescence) and anti-Akt (green fluorescence) antibodies. Values in merge panels indicate % of cells bearing both colors (approx. 150 cells bearing similar levels of Akt expression were scored in duplicate coverslips). After 24 h of infection, virus yield was determined by plaque assay, vesicular stomatitis virus (VSV) was employed as a negative control (e). Vero cells infected with JUNV at an MOI of 1 PFU/cell were treated at different times p.i. with 10 μM Ly294002, further incubated up to 24 h p.i. and virus yield quantified by plaque assay (f). Vero cells were infected with JUNV at an MOI of 10 PFU/cell in the presence or absence of 10 μM Ly294002, incubated at 37 °C and lysed by freeze and thawing at different times post-inoculation (g). Internalized virus in cell lysates was quantified by plaque assay. Vero cells treated or not with 10 μM Ly294002 were incubated with TRITC-labeled transferrin (SIGMA) and processed for IFA at 15 and 30 min post-contact (h). Vero cells previously incubated with 20 μg/ml transferrin (Tf) or 10 μM Ly294002 (Ly) or both transferrin and Ly294002 (Ly + Tf) were infected with approx. 150 PFU of JUNV and overlayed with plaquing medium (i). All experiments ($n = 3$) were performed on 24 h serum starved cultures and infection and/or treatment were carried out in serum free medium.

virus replication that accounts for the superinfection exclusion phenomenon observed for homologous or JUNV antigenically related viruses (Ellenberg et al., 2004). This restriction was due, at least in part, to an enhanced expression of TSG101, a protein involved in the vacuolar protein sorting machinery, that prevents budding of JUNV in persistently infected BHK-21 cells (Ellenberg et al., 2007). Taken into consideration that TSG101 is regulated by Akt through MDM2 phophorylation (Mayo and Donner, 2001), which in turn ubiquitinates the p53 tumor supression protein, tagging it for degradation and leading to an anti-apoptotic scenario (Zhou et al., 2001), it is tempting to speculate that activation and stabilization of MDM2 by PI3K/Akt pathway and TSG101, respectively, might be critical

to define the fate of JUNV multiplication in persistently infected cells.

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