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Extended stability of cyclin D1 contributes to limited cell cycle arrest at G1-phase in BHK-21 cells with Japanese encephalitis virus persistent infection

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There is increasing evidence that many RNA viruses manipulate cell cycle control to achieve favorable cellular environments for their efficient replication during infection. Although virus-induced G0/G1 arrest often delays early apoptosis temporarily, a prolonged replication of the infected virus leads host cells to eventual death. In contrast, most mammalian cells with RNA virus persistent infection often escape cytolysis in the presence of productive viral replication. In this study, we demonstrated that the extended endurance of cyclin D1 was clearly associated with the suppression of glycogen synthase kinase-3β (GSK-3β) expression in BHK-21 cells that are persistently infected with Japanese encephalitis virus (JEV). The G0/G1 arrest of these cells turned much loose compared to the normal BHK-21 cells with JEV acute infection. After cycloheximide treatment, cyclin D1 in the persistently infected cells lasted several hours longer than those in acutely infected cells. Furthermore, both $p21^{Cip1}$ and $p27^{Kip1}$, positive regulators for cyclin D1 accumulation in the nucleus, were suppressed in their expression, which contrasts with those in JEV acute infection. Inhibition of the GSK-3β by lithium chloride treatment rescued a significant number of cells from cytolysis in JEV acute infection, which coincided with the levels of cyclin D1 that escaped from proteolysis. Therefore, the limitation of G1/S arrest in the BHK-21 cells with JEV persistent infection is associated with the suppression of GSK-3β expression, resulting in the extended duration of cyclin D1.

Keywords: Japanese encephalitis virus, persistent infection, cell cycle, cyclin D1, GSK-3β

Introduction

Because viruses have to depend on the resources provided by the host cell, manipulation of the cell cycle by the infected virus should be one of the favorable strategies to reorganize a cellular environment that is supportive of the virus replication. This phenomenon has been most extensively demonstrated with various DNA viruses, as they replicate primarily in the nucleus of the host cell. Examples include small DNA viruses, such as the simian virus 40 (DeCaprio *et al.*, 1988; Fanning and Knippers, 1992), human papilloma virus (Werness *et al.*, 1990), and adenovirus (Howe *et al.*, 1990; Eckner *et al.*, 1994), and larger DNA viruses, like herpes viruses (Flemington, 2001). Although most RNA viruses replicate in the cytoplasm primarily, however, several RNA viruses also manipulate the cell cycle for their efficient replication in both the acute infections or the long-term persistent infections (Nascimento *et al.*, 2012). The list of RNA virus members intervening host cell cycle during infection includes several influenza virus species, coxsackievirus, hepatitis C virus, and coronaviruses (Luo *et al.*, 2003; Dove *et al.*, 2006; Yuan *et al.*, 2010; Kannan *et al.*, 2011). Japanese encephalitis virus (JEV) was added to this list recently as described in a neural progenitor cell study (Das and Basu, 2008). It reported that host cell cycle arrest in the G0/G1 phase was induced by the replication of mouse hepatitis virus or severe acute respiratory syndrome coronavirus (Yuan *et al.*, 2007; Zhirnov and Klenk, 2007), while infectious bronchitis virus induced G2/M phase arrest (Dove *et al.*, 2006). These observations implicate that many RNA viruses may have developed the capability to manipulate host cell cycles in order to maximize their competitiveness as a parasitic life form throughout millions of years.

JEV, a mosquito-borne flavivirus, is known to cause serious damage to the brain by inducing encephalitis during human infection with high mortality (Umenai *et al.*, 1985). JEV infection in humans can manifest a spectrum of diseases, from asymptomatic infection to a mildly febrile symptomatic illness, or to a life-threatening disease that affects the central nervous system (Vaughn and Hoke, 1992; Song *et al.*, 2012). JEV is usually cytolytic for susceptible cells; yet, persistent infection has been demonstrated in cell cultures (Schmaljohn and Blair, 1977, 1979), as well as in a mouse model (Mathur *et al.*, 1986). JEV persistence in the human nervous system occurs in approximately 5% of JEV-associated encephalitis cases, which suggests that JEV persistence might contribute to neural sequelae following the acute infection phase (Ravi *et al.*, 1993; Desai *et al.*, 1994).

The eukaryotic cell cycle is controlled at various biological checkpoints by cyclins, cyclin-dependent kinases (Cdk), and Cdk inhibitors (Lim and Kaldis, 2013). The key element in cell cycle regulation is the periodic synthesis and degradation of cyclins, protein factors that associate with and activate

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Cdks (Nascimento *et al.*, 2012). While the cyclin D-Cdk4/6 complex is responsible for G1-phase progression from quiescence, the cyclin E-Cdk2 complex is required for G1/S transition (Sherr, 1994; Obaya and Sedivy, 2002; Kim *et al.*, 2010). Hyperphosphorylation of the Rb protein by the cyclin E-Cdk2 complex in the late G1-phase allows E2F, a potent transcriptional activator for the genes causing cell proliferation, to be activated through dissociation from the phosphorylated Rb protein (Satyanarayana and Kaldis, 2009). Cdk inhibitors also play important roles in G1-phase progression by inhibiting active Cdk-cyclin complexes. Among more than 20 Cdk inhibitors, the Cip/Kip family, composed of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, has a wide spectrum of inhibitory effects on G1 Cdk-cyclin complexes (Nakayama and Nakayama, 1998). A recent report demonstrated that JEV induces G0/G1 arrest in the infected cells (Das and Basu, 2008). By doing so, JEV may obtain better chances for efficient replication and transcription (Feuer *et al.*, 2002; Luo *et al.*, 2003), translation (Rice *et al.*, 1985), and virion assembly (Lowe *et al.*, 1998; Lin and Lamb, 2000; Su *et al.*, 2002). Several reports also suggest that G1 cell cycle arrest may be undertaken by JEV to avoid the early apoptosis of infected cells and therefore gain sufficient time and resources for the viral life cycle (Gozlan *et al.*, 1998; Stewart *et al.*, 1999; Zhirnov and Klenk, 2007). Nevertheless, multiple reports have stated that most of the persistent infections of the cytolytic RNA viruses in cell cultures, including JEV, do not impose devastating cell death, even with the obvious replication of the viruses in host cells (Yoon *et al.*, 2006; Park *et al.*, 2013). It is therefore reasonable to ask whether there is any modification in the cell cycle control of the host cells with viral persistence turning the arrest loose, rescuing them from eventual cell death.

In previous studies we reported on the generation of mammalian cell lines with JEV persistent infection (Yoon *et al.*, 2006; Park *et al.*, 2013). In particular, the BHK-21 cells with JEV persistent infection were free from apoptotic cell death almost completely in the presence of apparent JEV replication, and the growth curve of these cells is comparable to that of normal BHK-21 cells (Park *et al.*, 2013). In this study, we employed two independent BHK-21 cell lines, cBS6-2 and cBS7-1, that are persistently infected with JEV as characterized previously (Park *et al.*, 2013). We attempted to investigate whether major cellular factors involved in the quiescence to G1/S progression are associated with the alleviation of cell cycle arrest during the JEV persistent infection. Our data shows that the expression and activity of glycogen synthase kinase-3β (GSK-3β) for the timely destruction of cyclin D1 was severely restrained. Furthermore, the expression of Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ were also significantly compromised. We believe this is the first study to suggest that the increased stability of cyclin D1, together with the suppression of the key inhibitors of Cdks in G1/S progression, contributes to alleviating the host cell cycle arrest in the BHK-21 cells with JEV persistent infection. This study could deepen our insight into cell cycle regulation and JEV persistence in mammalian cells.

Materials and Methods

Cells and viruses

Baby hamster kidney-21 (BHK-21; Korea Cell Line Bank) cells were maintained in a minimum essential medium (MEM; Gibco) containing 5% FBS (Gibco) and 100 units of penicillin-streptomycin (Gibco) per ml. Two persistently JEV-infected BHK-21 cells, cBS6-2 and cBS7-1, were chosen for this study. These cells have been described previously (Park *et al.*, 2013). All cells were grown at 37°C in a 5% CO_2 incubator. JEV K94P05 strain (provided by the Korean National Institute of Health) (Yoon *et al.*, 2006) was employed throughout this study.

Viable cell number counting

The cells were seeded in six-well plates, and viable cells were counted at different time intervals. The cells (10 μl/well) were mixed with trypan blue dye (10 μl), and 10 μl of this cell-dye mixture was loaded onto a hemacytometer, which was followed by microscopic observation.

Plaque formation assay

The virus was inoculated on a monolayer of naïve BHK-21 cells in 35 mm plates; an overlay medium, containing 5% FBS, 1% penicillin-streptomycin (10,000 U), 15% 5X MEM, 61% 1X MEM, and 1% agarose, was then applied. After 4 days of incubation, the cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 2 h and stained with 0.1% crystal violet.

Western blotting

For the intracellular protein extraction, virus- or mock-infected cells in a monolayer were lysed in an ice-cold M-PER buffer (Pierce-Thermo Fisher Scientific) with a protease inhibitor cocktail (Roche). The isolated proteins were separated with gradient polyacrylamide gel electrophoresis by using a Gradi-Gel II gradient analysis kit (Elpis biotech) and were transferred to a PVDF membrane (Millipore). After blocking with 5% skim milk in Tris-buffered saline-Tween 20 (TBST), the membrane was incubated with various primary antibodies specific to GSK-3β, Cdk2, Cdk4, Cdk6, cyclin E (Santa Cruz Biotechnology), $p27^{Kip1}$ (Cell Signaling Technology), cyclin D1, $p21^{Cip1}$ (BD), and β-actin (Neomarker). After the primary incubation, the membrane was washed in TBST once for 5 min and three times for 10 min. The membrane was then incubated with secondary antibodies (HRP-conjugated goat anti-mouse and -rabbit) for 1 h at room temperature. The membrane was washed again three times in TBST for 10 min, and the proteins were then visualized using a luminol-based chemiluminescent detection kit (AbFrontier-Young In Frontier).

Cell cycle analysis by flow cytometry

The nuclear DNA content was measured by performing propidium iodide (PI) staining and a fluorescence-activated cell sorting (FACS) analysis. After the adherent cells were harvested with trypsin treatment, they were briefly washed with PBS. The cells were fixed in 1 ml of cold 70% ethanol over-

night at 4°C and were resuspended in staining buffer [50 μg/ml PI (Sigma), 50 μg/ml RNase in PBS)] for 20 min at room temperature.

Statistical analysis

Statistically significant differences were determined by using Student's *t*-test in SPSS version 21.0 (SPSS). *P* values < 0.05 were considered statistically significant.

Results and Discussion

BHK-21 cells with JEV persistence produce infectious virus particles while demonstrating stable cell growth

In order to show the nature of JEV persistence in the cBS6-2 and cBS7-1 cells that we employed for this study, the same number of cBS6-2, cBS7-1, and normal BHK-21 cells was seeded in a six-well plate and the viable cells were counted every 24 h by using trypan-blue exclusion assay. Both the cBS6-2 and cBS7-1 cells revealed the retarded growth. The maximum number of viable cells reached at 4 days after seeding was 40–50% of the normal BHK-21 cells. Nevertheless, the overall cell growth of these two cell lines appeared quite stable, with no apparent cytolysis (Fig. 1A). The infectious JEV particles produced and released into the cell culture media were titrated by plaque formation assay on the normal BHK-21 cells. Both the cBS6-2 and cBS7-1 cells produced infectious JEV persistently during 5 days of the culture period (Fig. 1B). Along with the increase of the viable cell numbers in the given culture, the amount of produced virion also increased in parallel, demonstrating that most of the individual cells in each culture were in a virally persistent condition (Fig. 1C). The plaque morphologies of the infectious JEV from both the cBS6-2 and cBS7-1 cells appeared much smaller than those of wildtype JEV (Fig. 4D), implying that there was a notable attenuation in the virus replication and cytopathicity. These results were consistent with the observations reported previously (Yoon *et al.*, 2006; Park *et al.*, 2013) and confirmed that the persistent infected cell lines progress their cell cycle and produce infectious particles continuously, indicating apparent viral replication and gene expression without any additional treatment.

G1/S cell cycle arrest turned loose in the BHK-21 cells with JEV persistence, but not in the acute infection

Since the cBS6-2 and cBS7-1 cells showed steady cell growth while producing infectious JEV constantly, we analyzed the progress of the cell cycle phases in these cells by comparing them to normal BHK-21 cells with an acute wildtype JEV infection. When freshly seeded normal BHK-21 cells reached about 70% confluence, wildtype JEV was infected at 0.1 multiplicity of infection (MOI). The cells were then collected at 24, 30, and 36 h post-infection (p.i.) and stained with propidium iodide (PI) in the presence of RNase A. The reason for the latest time point for cell harvest at 36 h p.i. was to prevent misinterpretations that could be derived from the effect of contact inhibition from the full confluence of the cells. The cBS6-2 and cBS7-1 cells were collected and PI-stained at 60 h after seeding during the time point for active production of the virus particles, but prior to reaching contact inhibition. In the cell cycle analysis by flow cytometry, the cell numbers restrained in the G1 phase of the JEV acute infection increased gradually as the infection proceeded up to 36 h p.i. (Fig. 2A). Meanwhile, the number of cells accumulated in the G1 phase in the cBS6-2 and cBS7-1 cells was similar to that of 24 h p.i. of the acute infection. This type

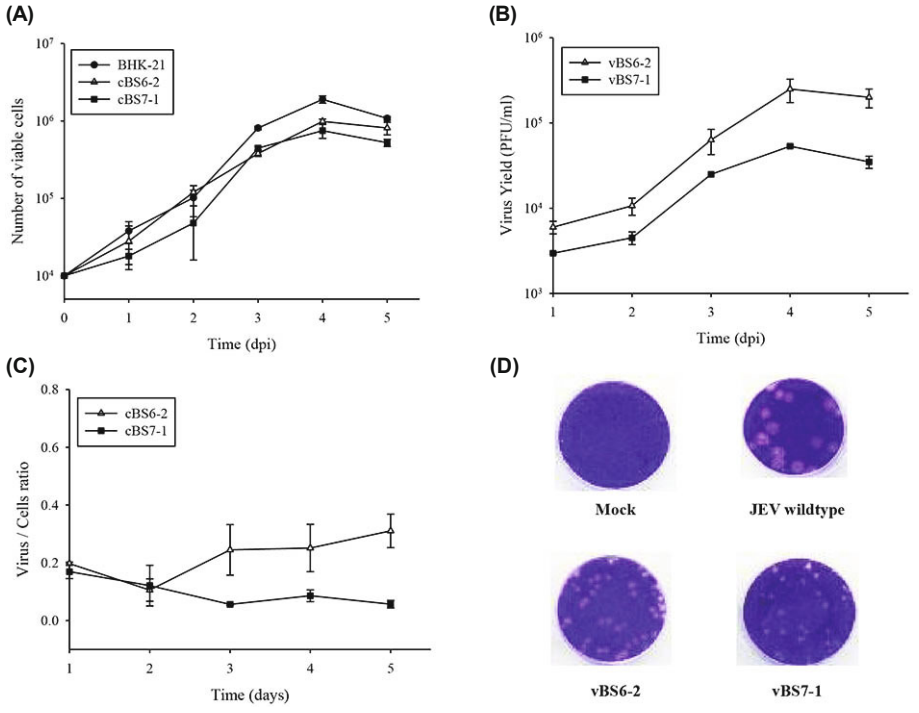

Fig. 1. Cell growth and progeny virus production of the persistently JEV-infected cells employed in this study. The same amounts of each cell line were seeded in six-well plates, followed by trypan blue exclusion and plaque-forming assay to confirm the number of viable cells (A) and virus yields (B). The virus production rates per cell from the persistent infected cell lines are displayed in panel (C). The mean ± SD of the three independent experiments is shown in these panels. (D) The plaque morphologies of the infectious JEV particles produced from the cBS6-2 and cBS7-1 cells were visualized by plaque-forming assay.

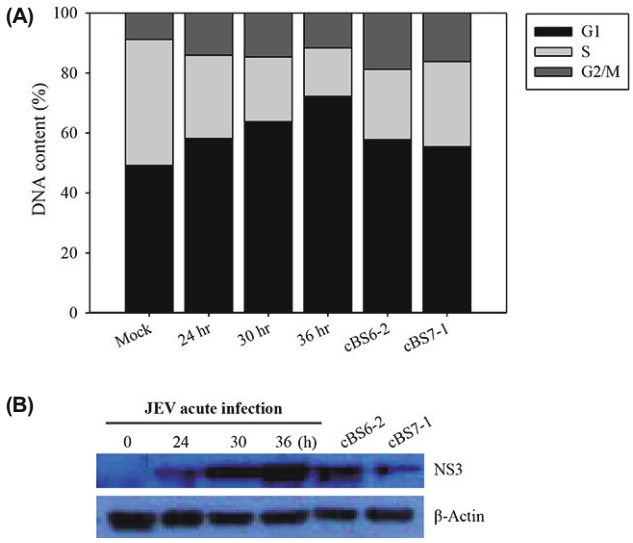

Fig. 2. Profiles of cell cycle progress and degree of virus replication in BHK-21 cells with JEV acute infection or JEV persistent infection. Cell cycle profiles (A) and a viral protein NS3 expression (B) of the acutely JEV-infected BHK-21 cells (0.1 MOI) at indicated time points (0 to 36 h p.i.) and persistently JEV-infected cBS6-2 and cBS7-1 cells. Cell cycle profiles of these cells were determined by flow cytometry analysis after propidium iodide staining. The JEV NS3 protein expression was analyzed by Western blotting using a NS3-specific antibody. β-Actin is shown as a loading control.

of cell cycle phase appeared consistent throughout the cell culture period, until the cells reached full confluence (data not shown). When we examined the degree of virus replication inside the host cells by looking at the NS3 expression of JEV, it was clear that the JEV infection induced the G1/S arrest, and this intensified as the infection proceeded (Fig. 2B). This result is consistent with the observation that JEV causes G1 arrest in neural progenitor cells, often followed by severe impairment in neurosphere formation in vivo (Das and Basu, 2008). It was also obvious that JEV replicates apparently in both the cBS6-2 and cBS7-1 cells. Interestingly enough, the cBS6-2 cells were suffered from G1/S arrest only slightly when the high level of NS3 expression in the cell was taken into account (Fig. 2B). Taken together, these results indicate that JEV acute infection obviously induces G1/S arrest in BHK-21 cells, while this adversity appeared clearly limited in cells with JEV persistent infection.

Cyclin D1, but not cyclin E, endures stably in BHK-21 cells with JEV persistent infection

The results of the cell cycle analysis indicated that the cell cycle arrest induced by JEV focused mainly on the G1/S progression (Fig. 2A). Therefore, we investigated the key molecules involved in the regulation of G1/S transition, especially for cyclins and Cdks. Freshly prepared normal BHK-21 cells were infected with wildtype JEV and harvested at 12, 24, and 36 h p.i. Intracellular proteins were extracted and subjected to Western blotting by using specific antibodies. As in previous experiments, the intracellular proteins of the cBS6-2 and cBS7-1 cells were obtained at 60 h after they were seeded. During the JEV acute infection, the amounts of cyclin D1 and cyclin E (both E1 and E2) declined rapidly as the infection proceeded, while no significant changes were noted in Cdk4/6 and Cdk2 expression (Fig. 3). Cyclin D1 in particular was barely detectable at 36 h p.i. In the cBS6-2 and cBS7-1 cells, meanwhile, cyclin D1 endured stably, with only a slight reduction that is comparable to the amount between 12–24 h p.i. in the acute infection (Fig. 3). It was noteworthy that the cyclin E in these cells was depleted completely. There were no significant changes identified in Cdk4/6 and Cdk2 expression, as in the acute infection. The coincidence of the G1-phase cyclin expressions of the G1/S arrest in the JEV acute infection indicates that the amount of cyclin D1 could be one of the main factors responsible for cell cycle progression from quiescence to G1/S transition in this condition (Figs. 2A and 3). Furthermore, the progressive transition of G1 to S-phase in the cBS6-2 and cBS7-1 cells in the complete absence of cyclin E indicates that cyclin D1 should be the one rescuing these cells from the G1 arrest. Since the primary antibody used in this experiment is specific for both cyclin E1 and E2, these results also confirm previous reports that E-type cyclins are not essential for G1/S transition and D- and A-type cyclins may compensate for the absence of E-type cyclins (Geng *et al.*, 2003; Satyanarayana and Kaldis, 2009).

Cyclin D1 in BHK-21 cells with JEV persistent infection demonstrate a higher stability than the one in acute infection

Cyclin D1 expression is essential for cell cycle progression from quiescence to G1/S transition by forming the Cdk4/6-cyclin D complex at an early G1-phase (Satyanarayana and Kaldis, 2009; He *et al.*, 2010). Considering the unstable nature of cyclin D1 and the finding that persistent infected cell lines are dependent on cyclin D1 for their cell cycle progression, we hypothesized that Cdk4/6-cyclin D1 complex in the cBS6-2 or cBS7-1 cells has a higher stability than in the acutely infected cells. Therefore, we examined the turnover rate of cyclin D1 after cycloheximide (CHX) treatment, which inhibits protein synthesis in the eukaryotic cells. Freshly prepared normal BHK-21 cells were infected with wildtype JEV at 0.1 MOI, and 20 μg/ml of CHX were treated for an hour

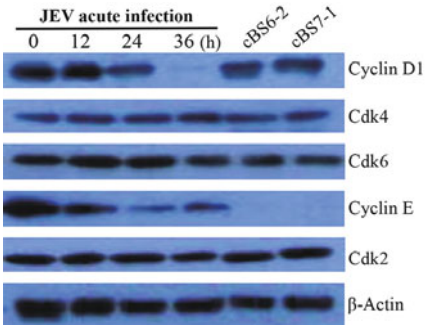

Fig. 3. Endurance of cyclin D1 and cyclin E and expression levels of Cdk proteins in G1-phase in BHK-21 cells with JEV acute infection or JEV persistent infection. Intracellular proteins were collected from the normal BHK-21 cells infected with 0.1 MOI of JEV at indicated time points or from the cBS6-2 and cBS7-1 cells with JEV persistent infection 60 h after they were seeded. The cell lysates were analyzed with Western blotting using the specific antibodies for cyclin D1, cyclin E, Cdk4, Cdk6, and Cdk2. β-Actin is shown as a loading control. The experiment was repeated three times, and the representative blots are shown.

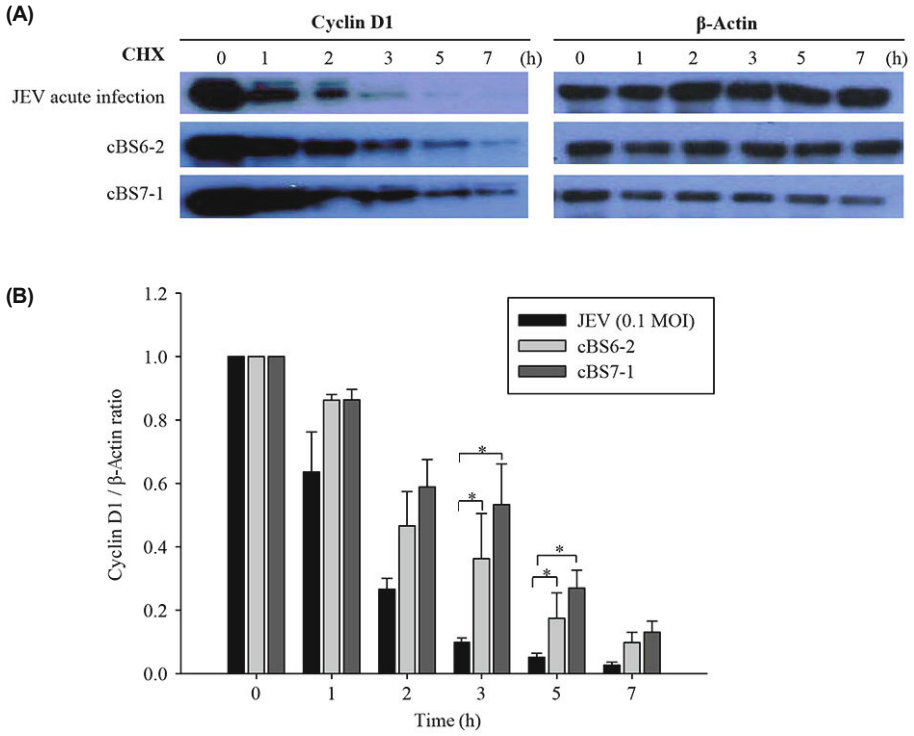

Fig. 4. Stability of cyclin D1 in BHK-21 cells with JEV acute infection or JEV persistent infection. (A) Cycloheximide (20 μg/ml, Sigma) was treated for an hour at 18 h p.i. of JEV (0.1 MOI) on normal BHK-21 cells. For the cBS6-2 and cBS7-1 cells, the same treatment was performed at 60 h after these cells were seeded. The intracellular proteins obtained at different time points were subjected to Western blotting by using cyclin D1-specific antibodies. β-Actin is shown as an internal control at the indicated time points. (B) The cyclin D1 endurance was quantitated by densitometry analysis using NIH Image J version 1.48z, and the activity was normalized at 0 h, which was arbitrarily set to a value of 1.0. The data represents one of three independent experiments (*statistically significant difference [$P < 0.05$]).

at 18 h p.i. The JEV-infected cells were harvested at various time points of up to 7 h after the CHX treatment, and the intracellular proteins were subjected to Western blotting to detect cyclin D1. Cyclin D1 endurance in the cBS6-2 and cBS7-1 cells was also analyzed in the same manner after CHX treatment for an hour. Cyclin D1 in the cells with JEV acute infection was destroyed completely within 5 h or less after CHX treatment (Fig. 4A). In the cBS6-2 and cBS7-1 cells, however, cyclin D1 molecules lasted up to 7 h or more. A quantitative analysis by densitometry revealed that the difference in the amounts of cyclin D1 left intact in the normal BHK-21 cells with the acute infection and in the cells with the persistent infection were statistically significant at 3 to 5 h after CHX treatment ($P < 0.05$) (Fig. 4B).

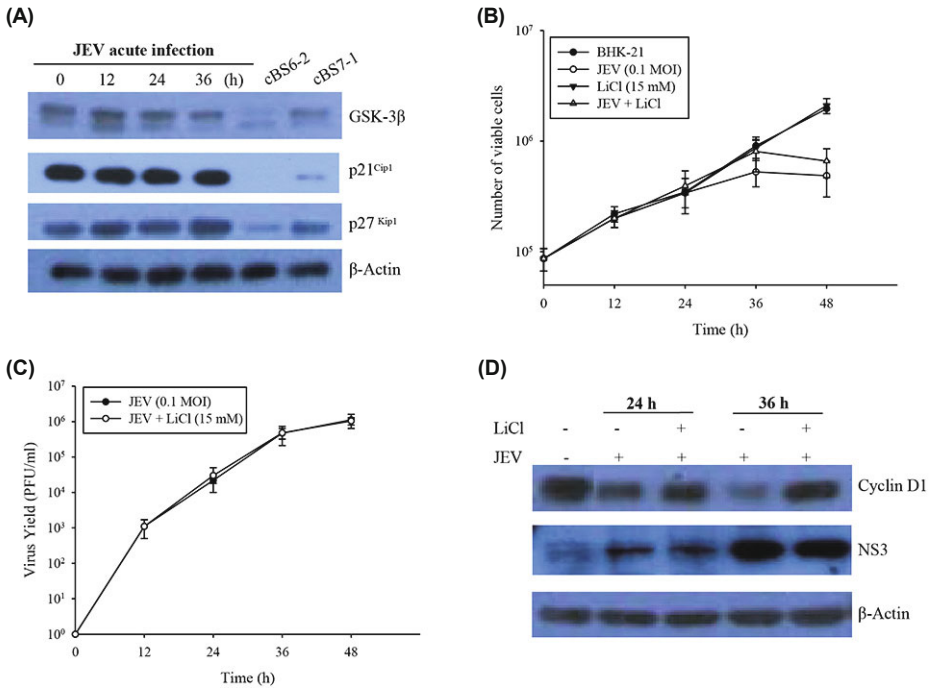

Fig. 5. Expressions of GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ and the correlation between the cell viability and GSK-3β activity in JEV acute infection. (A) Intracellular proteins were isolated from the BHK-21 cells infected with JEV (0.1 MOI) or from cBS6-2 and cBS7-1 cells at 60 h after they were seeded. The expression of GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ was examined by Western blotting by using specific antibodies. This experiment was repeated three times, and the representative blots are shown. (B) The viable cells were counted by trypan blue exclusion assay, and (C) the viral yields were determined by plaque-forming assay derived from the normal BHK-21 cells infected with JEV (0.1 MOI) that were exposed to 15 mM LiCl for 1 h at 12 h p.i. (D) After 24 and 36 h of JEV infection in the normal BHK-21 cells, the intracellular proteins were examined for cyclin D1 and JEV NS3 with Western blotting. The protein amounts were standardized, as shown, with β-actin, and the Western blotting data represents one of three independent experiments.

Expression of Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ and cyclin D1-degrading enzyme GSK-3β were all suppressed in BHK-21 cells with JEV persistent infection

Degradation of the cyclin D1 was triggered by phosphorylation of Thr286 by GSK-3β, followed by export to the cytoplasm and ubiquitin-dependent proteolysis (Diehl *et al.*, 1998). Furthermore, the Cdk inhibitor proteins $p21^{Cip1}$ and $p27^{Kip1}$ can contribute to the stability of cyclin D1-Cdk complex. In particular, $p21^{Cip1}$ inhibits the nuclear export of cyclin D1 mediated by GSK-3β (Alt *et al.*, 2002). Accordingly, we then examined the levels of GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ to determine whether these regulatory proteins play a role in extending cyclin D1 stability in the BHK-21 cells with JEV persistence.

Normal BHK-21 cells were infected with 0.1 MOI of wildtype JEV and were harvested at 0, 12, 24, and 36 h p.i. Both cBS6-2 and cBS7-1 cells that were persistently infected with JEV were also harvested at 60 h after they were seeded. All the cells were carefully monitored so that they would not be affected by contact inhibition from cell proliferation during the experiment. The intracellular proteins of these cells were obtained and then subjected to Western blotting by using the specific antibodies. The GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ levels in the normal BHK-21 cells remained unchanged throughout the acute JEV infection (Fig. 5A). In contrast, these three regulatory proteins were barely detectable in the cBS6-2 cells. Although the amount of these proteins in the cBS7-1 cells appeared to be slightly higher than that of cBS6-2, the overall suppression of GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ was quite obvious compared to that in the cells with JEV acute infection (Fig. 5A). Thus, it is very likely that the compromised levels of the GSK-3β, $p21^{Cip1}$, and $p27^{Kip1}$ may contribute to the increase of cyclin D1 stability in the cells with JEV persistent infection. However, although $p21^{Cip1}$ can facilitate cyclin D1 accumulation in the nucleus, the loss of both $p21^{Cip1}$ and $p27^{Kip1}$ does not abolish the nuclear import of cyclin D1 (Alt *et al.*, 2002). Accordingly, the positive effect of $p21^{Cip1}$ and $p27^{Kip1}$ suppression on the endurance of cyclin D1 in the cells with JEV persistent infection could not be ascertained exclusively by this study.

The phosphorylation and proteolytic turnover of cyclin D1 during the G1 and S phases were associated through the activity of GSK-3β (Diehl *et al.*, 1998). The increase of the cyclin D1 transcription and down-regulation of GSK-3β through Ras-dependent signaling pathways after mitogenic stimuli gave precise and fast control of the total amount of cyclin D1 protein, subsequently correlating with the Cdk4/6-cyclin D1 activity (Casanovas *et al.*, 2000). Thus, we attempted to evaluate the effect of GSK-3β activity on cyclin D1 endurance in JEV infection. Freshly prepared normal BHK-21 cells were infected with wildtype JEV, and then treated with 15 mM of GSK-3β inhibitor lithium chloride (LiCl) (Sohn *et al.*, 2014) for an hour at 12 h p.i. The number of viable cells, virus yields, and the amount of cyclin D1 in this acute infection were determined at various time points. The cells infected with JEV and treated with LiCl maintained their viability well compared to those that were not treated with LiCl after 24 h of infection; this difference was the most apparent at 36 h p.i. (Fig. 5B). No adverse effects were observed from the LiCl treatment of the cell growth or the virus yield in JEV infection (Fig. 5B and C). The amount of cyclin D1 decreased rapidly in the acutely infected cells that were not treated with LiCl, while that in the cells treated with LiCl maintained the level close to that of the uninfected cells (Fig. 5D). The difference in the amount of cyclin D1 between these cells was the most obvious at 36 h p.i. This observation of cyclin D1 levels is consistent with the results showing the increased cell viability in JEV acute infection after LiCl treatment (Fig. 5B).

In conclusion, the extended duration of cyclin D1 may unloose G1/S cell cycle arrest, and the limited expression or activity of GSK-3β contributes significantly to the cyclin D1 endurance in the BHK-21 cells with JEV persistent infection. The overall suppression of $p21^{Cip1}$ and $p27^{Kip1}$ levels could participate in this limitation of the early phase of cell cycle arrest. The detailed mechanisms of the suppression of these regulatory proteins and the cyclin E depletion in the cells with JEV persistent infection are the next topics to be addressed. Nevertheless, this is the first report that the limitation of cell cycle arrest in mammalian cells with flavivirus persistence is associated with the major players in G1/S progression. The observations in this study may provide an additional stepping stone to elucidating the exact mechanisms of the delicate balance between the persistence of lytic RNA viruses and host cell survival in relation to the cell cycle.

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