Inhibition of cell proliferation by SARS-CoV infection in Vero E6 cells

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Abstract

Severe acute respiratory syndrome (SARS) is caused by SARS-coronavirus (SARS-CoV). Infection of Vero E6 cells with SARS-CoV inhibits cell proliferation. Our previous study indicated that Akt, which is poorly phosphorylated in confluent cultures of Vero E6 cells, is phosphorylated and then dephosphorylated upon infection by SARS-CoV. In the present study, we showed that a serine residue of Akt was phosphorylated in Vero E6 cells in subconfluent culture and that Akt was dephosphorylated rapidly after SARS-CoV infection without up-regulation of its phosphorylation. Phosphorylation of glycogen synthase kinase-3β, which is one of the downstream targets of Akt, was prevented in SARS-CoV-infected cells. However, treatment with glycogen synthase kinase-3β small interfering RNA indicated that the glycogen synthase kinase-3β signaling pathway was not related to inhibition of cell proliferation. Treatment of Vero E6 cells with the phosphatidylinositol 3′-kinase/Akt inhibitor, LY294002, which induces dephosphorylation of Akt, inhibited cell proliferation. As shown in our previous studies, apoptosis occurred in virus-infected cells within 18 h postinfection. Cellular mRNA transcription, which was reported to be up-regulated in SARS-CoV-infected Caco-2 cells, was not up-regulated in virus-infected Vero E6 cells, partially as a result of apoptosis. These results suggested that inhibition of cell proliferation is regulated by both the phosphatidylinositol 3′-kinase/Akt signaling pathway and by apoptosis in SARS-CoV-infected Vero E6 cells. This is the first study to analyze SARS-CoV-induced cell growth inhibition.

Introduction

Severe acute respiratory syndrome (SARS) is a newly discovered infectious disease caused by a novel coronavirus, SARS coronavirus (SARS-CoV) (Marra, 2003; Rota, 2003). SARS spread from Guangdong province in China to more than 30 countries in late 2002, causing severe outbreaks of atypical pneumonia. Because both the virulence and the mortality rate of this virus are very high, it is important to understand the mechanisms of pathogenesis of SARS-CoV infection for the prevention of SARS.

Our recent studies indicated that signaling pathways are activated upon infection of confluent cultures of Vero E6 cells with SARS-CoV (Mizutani et al., 2004a,b,c). Mitogen-activated protein kinases (MAPKs), including p38, c-Jun N-terminal protein kinase (JNK) and extracellular signal-related kinase (ERK) 1/2, and their MAPKKs, are activated in SARS-CoV-infected Vero E6 cells (Mizutani et al., 2004b). In particular, p38 is involved in induction of apoptosis

because a p38 inhibitor was shown to partially prevent apoptosis induced by SARS-CoV infection. Signal transducer and activator of transcription (STAT)-3, which is ordinarily phosphorylated at a tyrosine residue in Vero E6 cells, is dephosphorylated by SARS-CoV-induced activation of p38 (Mizutani et al., 2004a). Akt, which has important antiapoptotic roles, is first phosphorylated at a single serine residue shortly after SARS-CoV infection, and subsequently dephosphorylated during the course of viral infection (Mizutani et al., 2004c). However, threonine phosphorylation of Akt is not detected, and glycogen synthase kinase (GSK)-3β, which is a downstream target of Akt, is slightly phosphorylated in virus-infected cells, suggesting that Akt is unable to prevent virus-induced apoptosis. Interestingly, both Akt and JNK signaling pathways are important for establishing persistent SARS-CoV infection of Vero E6 cells (Mizutani et al., 2005). The weak activation of Akt in cloned Vero E6 cells cannot allow escape from SARS-CoV-induced apoptosis. Thus, the signaling pathway of apoptosis is stronger than

the antiapoptotic pathway in virus-infected Vero E6 cells. Recent studies showed that expression of nucleocapsid, X1, and spike proteins of SARS-CoV can induce apoptosis in cells (He *et al.*, 2003; Surjit *et al.*, 2004; Chang *et al.*, 2004; Tan *et al.*, 2004). Interestingly, nucleocapsid protein induced apoptosis into COS-1 cells in the absence of growth factors, and both JNK and p38 MAPK were up-regulated in nucleocapsid expressing cells, whereas ERK1/2 and Akt were down-regulated (Surjit *et al.*, 2004).

Akt activated by phosphatidylinositol 3′-kinase (PI3K) regulates cell proliferation, the cell cycle and cell survival. A number of proteins, such as Bad, caspase-9 and GSK-3β, have been identified as downstream targets of Akt (Cardone *et al.*, 1998; Pap & Cooper, 1998; Vanhaesebroeck & Alessi, 2000). These proteins are inactivated when phosphorylated by Akt. Activation of Akt by infection with a number of viruses has been reported previously. LMP-1 protein of Epstein–Barr virus has been shown to bind to the p85 subunit of PI3K, resulting in activation of both PI3K and Akt (Dawson *et al.*, 2003). The middle T antigen of murine polyomavirus and the X protein of hepatitis B virus also bind to the p85 subunit of PI3K, leading to activation of Akt (Whitman *et al.*, 1985; Dahl *et al.*, 1998; Lee *et al.*, 2001). Activation of the PI3K/Akt signaling pathway by a variety of viruses is thought to be involved in the establishment of latent and chronic infections by allowing virus-infected cells to escape from apoptosis. Activation of the PI3K/Akt signaling pathway may lead to a delay in apoptosis of host cells, and the virus life cycle might be completed before apoptotic cell death of the host cells. Subsequent apoptosis facilitates the spread of the virus (Roulston *et al.*, 1999). As described above, the PI3K/Akt signaling pathway also promotes cell proliferation. Mirza *et al.* (2004) reported that the PI3K/Akt signaling pathway cooperates with the ERK signaling pathway to promote cell cycle progression in both normal and cancer cells. GSK-3β is a ubiquitously expressed protein-serine/threonine kinase and its activity is inhibited by Akt phosphorylation (Cross *et al.*, 1995). In addition, GSK-3 is now a well-known key component in a large number of cellular processes and disease states, including p53, hypoxia, prions and endoplasmic reticulum stress (Loberg *et al.*, 2002; Song *et al.*, 2002; Watcharasit *et al.*, 2002; Perez *et al.*, 2003). The effects of activation of GSK-3β are mediated through phosphorylation and proteolytic turnover of cyclin D1 (Diehl *et al.*, 1998), resulting in the induction of cell cycle arrest via prevention of retinoblastoma tumor suppressor protein (Rb) hyperphosphorylation.

In the present study, we found that cell proliferation was completely abolished when growing Vero E6 cells were infected with SARS-CoV. Furthermore, we showed that Akt was dephosphorylated without any increase in its phosphorylation upon SARS-CoV infection in subconfluent cultures of Vero E6 cells, in contrast to the tentative up-regulation of phosphorylation of Akt prior to dephosphorylation in confluent cultures of these cells. In our previous study, we showed that caspase-3, which is one of the key factors in apoptosis, was activated within 18 h postinfection (p.i.) in virus-infected cells. Thus, both acute dephosphorylation of Akt and apoptotic events are likely to contribute to the inhibition of cell proliferation of SARS-CoV infected Vero E6 cells.

Materials and methods

Cells and virus

Vero E6 cells were subcultured routinely in 75-cm³ flasks in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) supplemented with 0.2 mM L-glutamine, 100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 5% (volume in volume, v/v) fetal bovine serum (FBS), and maintained at 37 °C in an atmosphere of 5% CO_2. For use in the experiments, the cells were split once on to 6-, 24- and 96-well tissue culture plate inserts and cultured until they reached 30% or 100% confluence (referred to as subconfluent and confluent, respectively). In the case of subconfluent cell culture, DMEM containing 5% FBS was used. The PI3K inhibitor, LY294002 (Cell Signaling Technology Inc., Beverly, MA), was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. DMSO alone was used as a control. SARS-CoV, which was isolated as Frankfurt 1 and kindly provided by Dr. J. Ziebuhr, was used in the present study. Infection was usually performed at a multiplicity of infection of 10.

Western blotting

After virus infection, whole-cell extracts were electrophoresed on either 10% or 10–20% gradient polyacrylamide gels, and transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). In the present study, we applied two sets of samples to polyacrylamide gels, and the membranes were either divided into two halves after blotting using a Proto-Blot II AP system (Promega Co., Madison, WI), or they were examined once using a LumiGLO Elite chemiluminescent system (Kirkegaard and Perry Laboratories, Gaithersburg, ML) and then stripped using Restore western blot stripping buffer (Pierce, Rockford, IL) for second detection. The following antibodies, obtained from Cell Signaling Technology Inc., were used in the present study at a dilution of 1 : 1000: rabbit antiphospho Akt (Ser473), rabbit antiphospho Akt (Thr308), rabbit anti-Akt, rabbit antiphospho GSK-3αβ rabbit antiphospho GSK-3β, rabbit anti-GSK-3β, rabbit antiphospho STAT3 (Tyr-705) antibody, rabbit antiphospho STAT3 (Ser-727) antibody, rabbit anti-p38 MAPK (Thr180/Tyr182) antibody, rabbit anti-p38 MAPK antibody,

rabbit antiphospho p44/42 MAPK (Thr202/Tyr204) (= ERK1/2) antibody, rabbit anti-p44/42 MAPK (= ERK1/2) antibody, rabbit antiphospho SAPK/JNK (Thr183/Tyr185) antibody, rabbit antiphospho Rb (Ser795), rabbit antiphospho Rb (Ser807/811) and mouse anti-Rb monoclonal antibody. Mouse anti-STAT3 antibody (diluted 1 : 2500) was obtained from BD Biosciences (Franklin Lakes, NJ). Mouse anti-β-Actin antibody was purchased from Sigma and used at a dilution of 1 : 5000. Rabbit anti-SARS nucleocapsid and membrane antibodies were described previously (Mizutani *et al.*, 2004b).

In vitro GSK-3 activation assay

Vero E6 cells were treated with or without epidermal growth factor (EGF) and LY294002 for 10 min, and then cell extracts were obtained using the lysis buffer supplied in the Akt kinase assay kit (Cell Signaling Technology Inc.). Selective immunoprecipitation of Akt was performed using immobilized Akt antibody. After incubation of immunoprecipitated Akt in kinase buffer containing GSK-3β fusion protein and ATP, GSK-3β phosphorylation was analyzed by western blotting using antiphospho GSK-3αβ antibody.

siRNA against GSK-3β

Both GSK-3β small interfering RNA (siRNA) and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Each siRNA (final concentration 100 nM) was transfected into 30% confluent Vero E6 cells in 96-well plates using the Magnetofection system (OZ Biosciences, Marseille, France) combined with FuGene 6 transfection reagents (Roche, Indianapolis, IL). Efficiency of transfection was determined using fluorescein-labeled Luciferase GL2 duplex (Dharmacon, Lafayette, CO), and we usually obtained nearly 100% transfection efficiency.

RT-PCR

Vero E6 cells were inoculated with SARS-CoV at a multiplicity of infection of 10. Total RNA was extracted from SARS-CoV-infected cells at 24 h p.i. and from mock-infected cells with Isogen (Nippon Gene, Tokyo, Japan). RNAs were reverse-transcribed using SuperScript III (Invitrogen, Carlsbad, CA) and random primers. Semiquantitative PCR amplification was performed using High Fidelity Platinum Taq DNA polymerase (Invitrogen) for 15 to 35 cycles. PCR primers were determined based on sequences with the accession numbers given in the report by Cinatl *et al.* (2004). Primers used in this study were for eukaryotic translation initiation factor 3, subunit 6 (forward 5′-TAATGCAATTCAGACAATGTGTCC-3′, reverse 5′-CTTCT GGAGTCATGTTCAATTTATCTGCC-3′), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5′-TGATG

ACATCAAGAAGGTGG-3′, reverse 5′-AGCTAGCTAATAG GACTCACTATAGGGTTACTCTCCTTGGAGGCCATGT-3′ including promoter sequence of T7 RNA polymerase), Ets variant gene 5 (forward 5′-CATGGCTACTCTTGGGAACC ACCCGGCC-3′, reverse 5′-ATGTACAACTCAGGTGCAAA TGTTCC-3′), zinc finger protein 384 (forward 5′-CTCTCT CCTTCTCTGCCTACAAAGACCC-3′, reverse 5′-GAAATG GTTACAACAAAGATGGCC-3′), core promoter element-binding protein (forward 5′-GCCGGGAGCCACGGGATTG GGCC-3′, reverse 5′-CGTTTACCTGTTGCCAGTACTCCT CC-3′), Von Hippel–Lindau syndrome (forward 5′-GAC GGCTGGCATGGTGGCTCCC-3′, reverse 5′-ACTCATGGC TCACTGCAGCCTCC-3′), transcription factor 8 (forward 5′-AACCTGTATGCTGTGATTCC-3′, reverse 5′-TCCCAAT TGTACAGCTTAAGAATAAACC-3′), trinucleotide repeat-containing 4 (forward 5′-TCAGCCACAGAGACCCTCCT CC-3′, reverse 5′-AGAAATTAAGAGAGAGAAAAAAATCC-3′), trichorhinophalangeal syndrome 1 (forward 5′-GCTTC AGTTTCATTGTTAACGGGCC-3′, reverse 5′-AAATATTG TTGTTTGGGGGAATCTCC-3′), inhibition of DNA-binding 4 (forward 5′-TATAGATAAATGAGTGACATTTCAT ACC-3′, reverse 5′-TTAAGGATCCAAGCATTTCCTCATCC C-3′), c-fos (forward 5′-CAGAGAGGAGAAACACATCTTC CC-3′, reverse 5′-GATACAATTTGAAAATATCCAGCACC-3′), Friend leukemia virus integration 1 (forward 5′-GACC-GAGTCGTCCATGTACAAGTACCC-3′, reverse 5′-AAAAC GTACAGCTTCTTTCTCAGAACC-3′), early growth response 1 (forward 5′-TGCAATTGTGAGGGACATGCTCA CC-3′, reverse 5′-AGCGCATTCAATGTGTTTATAAGCC-3′) and synoviral sarcoma, X breakpoint 4 (forward 5′-CT GTATTTTCTTGCAGTGTGCC-3′, reverse 5′-AACAGAGT GAGGGGGGCTTGACC-3′). The PCR products were confirmed to be of the correct sizes.

Results

Regulation of cell proliferation upon SARS-CoV infection

Mouse hepatitis virus (MHV), a prototype coronavirus, has been shown to arrest the cell cycle at G0/G1 phase (Chen *et al.*, 2004b). Therefore, we investigated whether SARS-CoV infection suppresses cell proliferation. Subconfluent Vero E6 culture cells were infected with SARS-CoV and the cell number was counted at 24 h p.i. As shown in Fig. 1(a,b), the number of virus-infected cells was not increased at 24 h p.i., whereas that of mock-infected cells was increased by 1.8-fold.

Dephosphorylation of a serine residue of Akt by SARS-CoV infection

As shown in our recent study (Mizutani *et al.*, 2004), a serine residue of Akt was phosphorylated from 8 to 18 h p.i. in

confluent Vero E6 cell cultures, and then dephosphorylated at 24 h p.i.; we concluded that Akt phosphorylation in SARS-CoV-infected cells had low activity (Mizutani *et al.*, 2004). Thus, phosphorylation followed by dephosphoryla-

tion of Akt occurred during the course of virus infection. However, it was still unclear whether virus infection was actually responsible for phosphorylation and/or dephosphorylation of Akt.

To investigate the phosphorylation level of Akt in growing Vero E6 cells, subconfluent cell cultures were used in the present study. As shown in Fig. 2b (mock infection lane), a serine residue of Akt was highly phosphorylated in subconfluent cultures. To examine whether the phosphorylated Akt was up- or down-regulated in subconfluent cell cultures upon SARS-CoV infection, a virus-infection time-course experiment was performed. Cells in subconfluent cultures were infected with the virus at a multiplicity of infection of 10 and western blot analyses were performed using antibodies to viral and cellular proteins at 7, 16 and 23 h p.i. Protein from mock-infected cells was obtained at 7 h. The time-dependent changes of (phospho-)protein level, such as viral nucleocapsid and membrane proteins, the kinetics of MAPKs, including p38, JNK and ERK 1/2, and STAT-3 in virus-infected subconfluent Vero E6 cells were similar to those in confluent cells after viral infection (Fig. 2a,b; Mizutani *et al.*, 2004b). As shown in Fig. 2a, only the kinetics of Ser473-phosphorylation of Akt were different. Akt, which was always phosphorylated in subconfluent cells, was dephosphorylated at 16 and 23 h p.i. On the other hand, Akt in mock-infected cells was continuously phosphorylated from 7 to 24 h p.i. (data not shown; Fig. 2c). We measured the
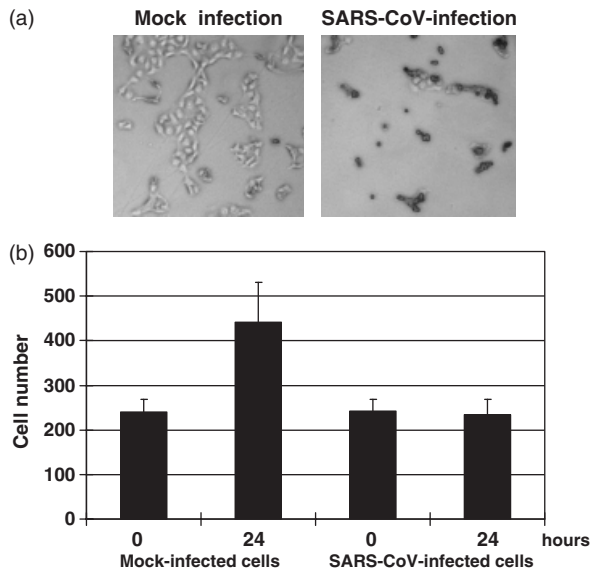

Fig. 1. Prevention of cell proliferation by severe acute respiratory syndrome coronavirus (SARS-CoV) infection. Subconfluent Vero E6 cells were infected with SARS-CoV at a multiplicity of infection of 10. Cell numbers of mock- and virus-infected cells were counted at 24 h postinfection.
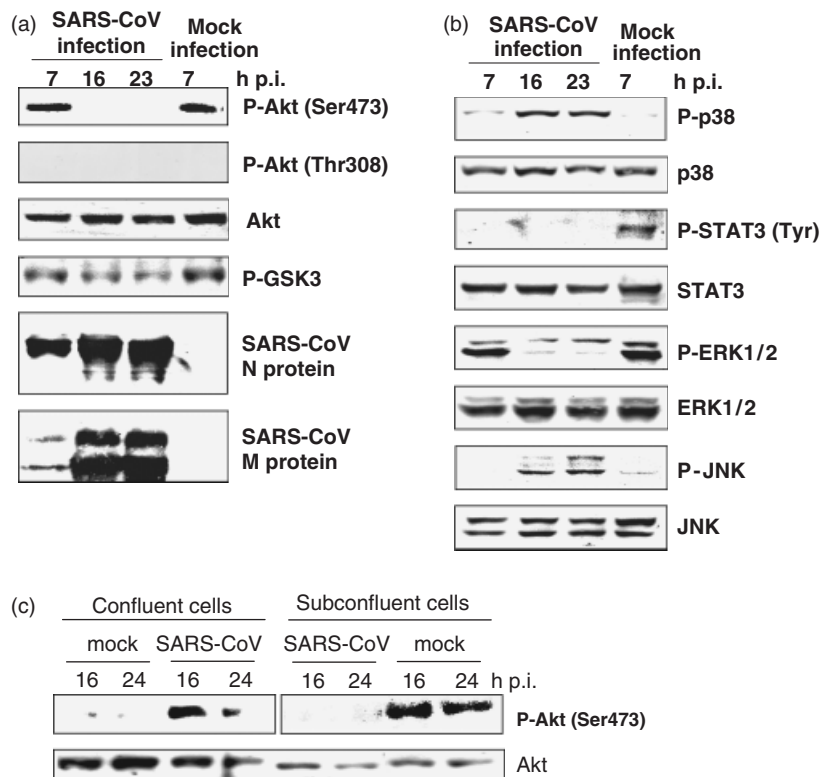

Fig. 2. Acute dephosphorylation of Akt serine by severe acute respiratory syndrome coronavirus (SARS-CoV) infection. (a,b) Subconfluent Vero E6 cells were infected with SARS-CoV at a multiplicity of infection of 10 and then western blot analysis was performed using anti-Akt, glycogen synthase kinase (GSK)-3β, viral proteins, p38, signal transducer and activator of transcription (STAT)3, extracellular signal-related kinase (ERK)1/2 and c-Jun nucleocapsid-terminal protein kinase (JNK) antibodies. (c) Confluent and subconfluent cells were infected with SARS-CoV at a multiplicity of infection of 10 and western blot analysis was performed. Proteins equivalent to same cell number were applied to the gel. p.i., postinfection.

densities of Ser473-phosphorylated Akt of mock-infected subconfluent cells and SARS-CoV-infected confluent cells at 16 h p.i. (Fig. 2c) using the LAS-3000 mini system (Fuji Photo Film Co. Ltd., Tokyo, Japan). The amount of phosphorylated Akt of mock-infected subconfluent cells was 4.8-fold higher than that of SARS-CoV-infected confluent cells. GSK-3β was also dephosphorylated in SARS-CoV-infected subconfluent cells. We next investigated whether Akt was phosphorylated in virus-infected subconfluent cell cultures before 7 h p.i. The phosphorylation level of Akt-serine was not up-regulated at 2, 4 or 6 h p.i. (data not shown). The phosphorylation of GSK-3 were also similar at 2 and 6 h p.i. (data not shown). In addition, phosphorylation level of Akt kinase did not significantly change from 7 to 23 h p.i. (data not shown). These results suggested that SARS-CoV infection induces dephosphorylation of a serine residue of Akt in subconfluent cultures, without tentative up-regulation of phosphorylation prior to dephosphorylation.

Activity of Akt in subconfluent cells

To investigate whether Akt serine phosphorylation represented a biologically active kinase in subconfluent cells, we examined the *in vitro* kinase activity of phosphorylated Akt. Subconfluent Vero E6 cells treated with EGF for 10 min or LY294002 for 1 h were lysed and Ser473-phosphorylated Akt in the cell lysate was precipitated with anti-Akt antibody. GSK-3β protein was added to the immunoprecipitated Akt with ATP, and western blotting was performed using antiphosphorylated GSK-3α/β antibody. As shown in Fig. 3, the level of phosphorylation of GSK-3 in EGF-treated cells was higher than that in nontreated cells. On the other hand, phosphorylation of GSK was not detected in cells treated with the PI3K inhibitor, LY294002. These results strongly suggested that Akt in subconfluent Vero E6 cell cultures was phosphorylated mainly at serine residues and had kinase activity.

Regulation of cell proliferation by Akt

The level of phosphorylated GSK-3β was decreased in virus-infected cells, possibly as a result of down-regulation of Akt
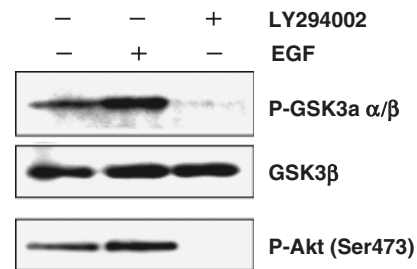
Fig. 3. Phosphorylation status of Akt in subconfluent cells. Epidermal growth factor and LY294002 were added to subconfluent Vero E6 cells for 10 min and 1 h, respectively. The cell lysates were precipitated using anti-Akt antibody. Glycogen synthase kinase (GSK)-3β protein was added to the immunoprecipitated Akt with ATP, and western blot analysis was performed using antiphosphorylated GSK-3α/β and anti-phosphorylated Akt (Ser) antibodies.

activation (Fig. 2a). Because one of the important roles of Akt is in cell cycle regulation by preventing GSK-3β-mediated phosphorylation and degradation of cyclin D1 (Diehl *et al.*, 1998), we examined whether GSK-3β regulates the proliferation of Vero E6 cells. Vero E6 cells were transfected with GSK-3β siRNA, and western blot analysis was performed 48 h later to detect the total amount of GSK-3β protein. As shown in Fig. 4a, the total amount of GSK-3β was reduced markedly by the siRNA, and the level of phosphorylated GSK-3β was reduced. However, GSK-3β siRNA-treated cells exhibited similar growth to those treated with control siRNA (Fig. 4b). The total amount of Rb and phosphorylated Rb was not affected by GSK-3β siRNA. The hyperphosphorylation form of Rb is known to be phosphorylated at least at Ser795, Ser807, and Ser811 (Faenza *et al.*, 2000; Jiang, 2002; Shibata & Nakamura, 2002), and hyperphosphorylation of Rb at Ser795 to release E2F is a critical step in the G1–S transition (Harbour & Dean, 2000).

We next investigated whether inhibition of the PI3K/Akt signaling pathway inhibits cell proliferation of Vero E6 cells. As shown in Fig. 5, complete inhibition by the PI3K inhibitor, LY294002, was observed at 48 h. We confirmed that serine residues of Akt were dephosphorylated in LY294002-treated cells in our previous study. These results
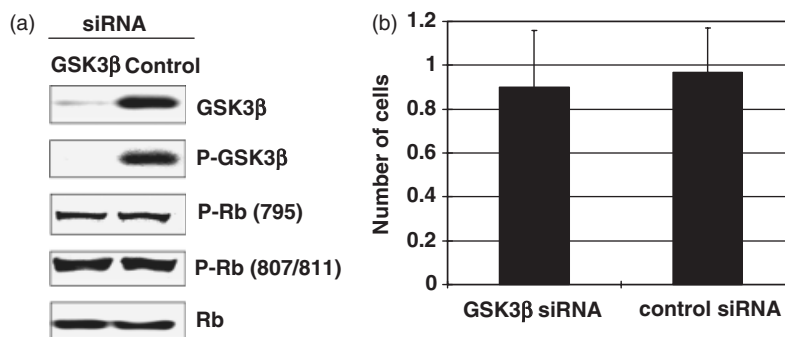
Fig. 4. Relationship between glycogen synthase kinase (GSK)-3β and Rb in cell proliferation. (a) GSK-3β small interfering RNA (siRNA) was transfected into subconfluent Vero E6 cells for 48 h and then western blot analysis was performed. (b) Cell number was determined using a WST-1 cell counting kit (Dojin, Kumamoto, Japan).

suggested that activation of Akt in subconfluent Vero E6 cells plays important roles in cell proliferation, and that down-regulation of Akt activity in SARS-CoV-infected cells prevents cell proliferation.

Down-regulation of cellular mRNAs in virus-infected cells

Although LY294002 inhibits proliferation of Vero E6 cells, apoptotic cell death was not induced by treatment with this
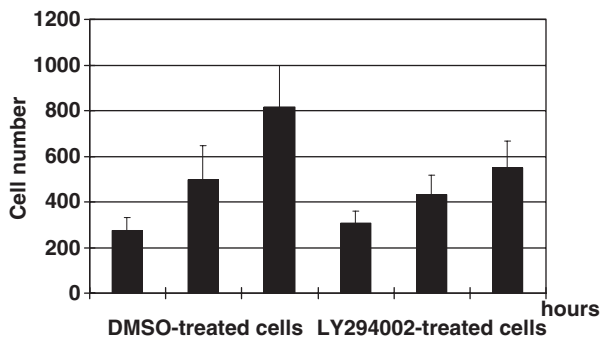
Fig. 5. Prevention of cell proliferation by LY294002 treatment. Subconfluent Vero E6 cells were treated with LY294002 at a concentration of 10 μM. Cell numbers of mock- and LY294002-treated cells were counted at 24 and 48 h postinfection.

agent (10 μM) for 48 h by western blot analysis using anti-cleaved caspase-3 (data not shown). As indicated in our previous study, apoptotic events progress in virus-infected cells. Therefore, these findings strongly suggested that both events Akt-dephosphorylation and apoptosis are responsible for the inhibition of cell proliferation in SARS-CoV-infected cells. We feel that transcriptional activity in virus-infected cells is important for understanding the viability of cells. Cinatl *et al.* (2004) demonstrated that SARS-CoV infection regulates cellular gene expression in a SARS-CoV-permissive cell line, Caco-2, at 24 h p.i. using high-density oligonucleotide arrays. We focused on up-regulated genes related to transfection factors, because several signaling pathways, which generally play important roles in the activation of transcription factors, are activated in SARS-CoV-infected Vero E6 cells. We selected 14 genes (including the housekeeping gene, GAPDH) that were shown previously to be up-regulated by several stimuli (Cinatl *et al.*, 2004), and semiquantitative reverse transcription PCR (RT-PCR) was performed using total RNA extracted from virus-infected confluent Vero E6 cells at 24 h p.i. The amount of total RNA was adjusted by the level of ribosomal RNA (Fig. 6s). Figure 6(b,c) shows genes that were down-regulated and that showed similar expression upon SARS-CoV-infection compared to mock-infected cells, respectively. Eukaryotic translation initiation factor 3, Ets variant gene 5, Zinc finger protein
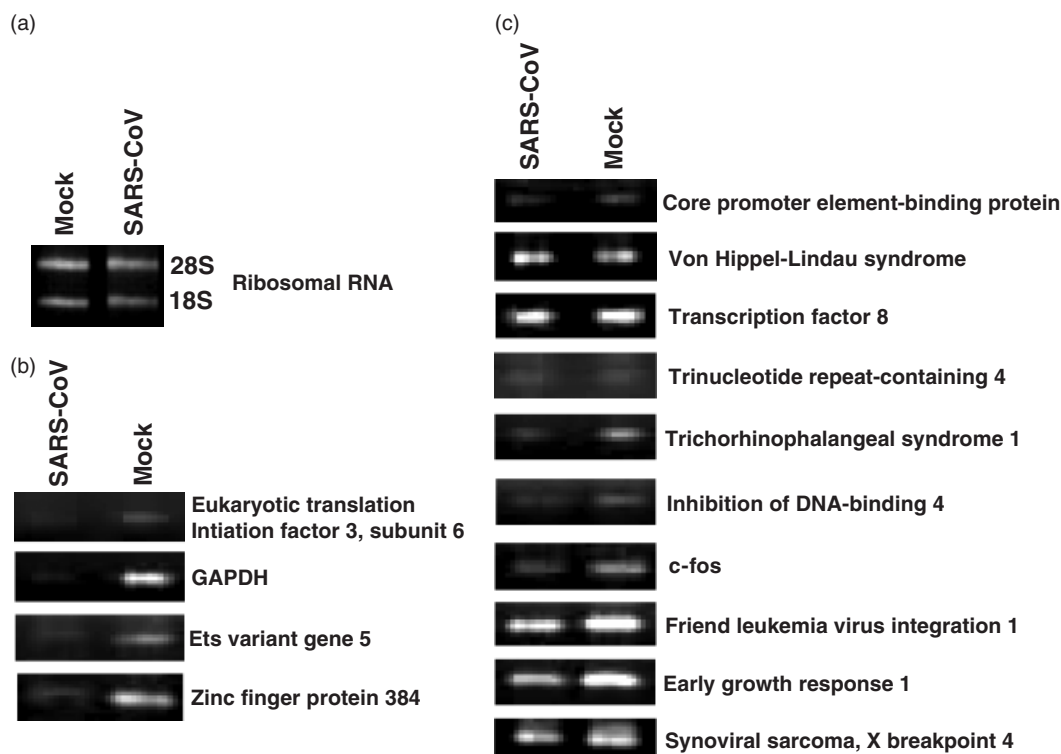
Fig. 6. Semiquantitative reverse transcription PCR (RT-PCR) of cellular transcripts. Total RNAs were extracted from virus-infected and mock-infected confluent Vero E6 cells at 24 h postinfection. Transcripts of cellular genes were assessed for their relative abundance by RT-PCR.

384 and GAPDH were down-regulated in SARS-CoV-infected cells. These results suggested that transcriptional shut-off occurred in particular (or in many) cellular mRNAs due to SARS-CoV infection, and that virus-infected cells are not proliferative due to Akt dephosphorylation and apoptosis.

Discussion

In the present study, we focused on the different kinetics of the phosphorylation status of Akt in subconfluent Vero E6 cells upon SARS-CoV infection. Akt-phosphorylation is up- and down-regulated by virus infection in the confluent cells, as shown in our previous report (Mizutani *et al.*, 2004), but is down-regulated by virus infection in subconfluent cells. Therefore, one of the aims of the present study was to determine whether SARS-CoV has the potential for up- or down-regulation of Akt-phosphorylation in Vero E6 cells. Akt was phosphorylated in growing mock-infected cells (i.e. 30% confluency), whereas it was dephosphorylated in confluent cell cultures. An Akt-binding protein, carboxyl-terminal modulator protein (CTMP), is a well-known negative regulatory component of the Akt (Maira *et al.*, 2001). CTMP, which binds to Akt, reduces the activity of Akt by inhibiting phosphorylation on serine and threonine residues. We found that the level of CTMP was below the limit of detection at 0–24 h p.i. in subconfluent virus-infected cells (data not shown), suggesting that dephosphorylation of Akt in subconfluent cell cultures upon viral infection was not regulated by CTMP. Although we obtained proteins from SARS-CoV-infected cells at different time points from 5 to 24 h p.i. in at least six experiments and performed western blot analysis using anti-phospho Akt (Ser) antibody, we were not able to find up-regulation of Akt-phosphorylation in SARS-CoV-infected subconfluent Vero E6 cells. Therefore, we concluded in this study that SARS-CoV has a potential of down-regulation of Akt-phosphorylation. It may be possible that SARS-CoV infection induces activation of Akt phosphatase(s).

In our previous study, we showed that apoptotic events, such as caspase-3 activation and DNA ladder formation, were detected in SARS-CoV-infected Vero E6 cells at 18 and 24 h p.i., respectively (Mizutani *et al.*, 2004b,c). We could not find any up-regulated genes in SARS-CoV-infected Vero E6 cells, in contrast with SARS-CoV-infected Caco-2 cells, in which several genes were demonstrated to be up-regulated (Cinatl *et al.*, 2004). This may have resulted from the faster progression of apoptosis and more rapid production of virus particles in infected Vero E6 as compared with Caco-2 cells. Thus, transcriptional shut-off in virus-infected cells may prevent cell proliferation with dephosphorylation of Akt.

In conclusion, the results of the present study suggested that SARS-CoV-infection affected Akt signaling pathways and gene expression. Down-regulation of Akt activity and the processing of apoptotic events in virus-infected cells may prevent cell proliferation. We assume that these phenomena are at least partly involved in the pathogenesis of SARS-CoV infection.

Acknowledgements

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