p21-activated Kinase 1 Is Activated through the Mammalian Target of Rapamycin/p70 S6 Kinase Pathway and Regulates the Replication of Hepatitis C Virus in Human Hepatoma Cells

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Cellular mechanisms that regulate the replication of hepatitis C virus (HCV) RNA are poorly understood. p21-activated kinase 1 (PAK1) is a serine/threonine kinase that has been suggested to participate in antiviral signaling. We studied its role in the cellular control of HCV replication. Transfection of PAK1-specific small interfering RNA enhanced viral RNA and protein abundance in established replicon cell lines as well as cells infected with chimeric genotype 1a/2a HCV, despite reducing cellular proliferation, suggesting specific regulation of HCV replication. PAK1 knockdown did not reduce interferon regulatory factor 3-dependent gene expression, indicating that this regulation is independent of the retinoic acid-inducible gene I/interferon regulatory factor 3 pathway. On the other hand, LY294002 and rapamycin abolished PAK1 phosphorylation and enhanced HCV abundance, suggesting that the mammalian target of rapamycin (mTOR) is involved in PAK1 regulation of HCV. Small interfering RNA knockdown of the mTOR substrate p70 S6 kinase abrogated PAK1 phosphorylation and enhanced HCV RNA abundance, whereas overexpression of a constitutively active alternate substrate, eukaryotic translation initiation factor 4E-binding protein 1, increased cap-independent viral translation and viral RNA abundance without influencing PAK1 phosphorylation. Similar data indicated that mTOR is regulated by both phosphatidylinositol 3-kinase/Akt and ERK. Taken together, the data indicate that p70 S6 kinase activates PAK1 and contributes to phosphatidylinositol 3-kinase and ERK-mediated regulation of HCV RNA replication.

Hepatitis C virus (HCV) is a globally important cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Approximately 170 million people are estimated to be infected with the virus worldwide. Classified within the family Flaviviridae, HCV has a positive sense single-stranded RNA genome about 9.6 kb in length. The precursor polyprotein encoded by the large open reading frame that spans much of the genome is processed into at least 10 proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by cellular and viral proteases. Up to 50–80% of patients infected with HCV fail to eliminate the virus, leading to long-term viral persistence and an increased risk of liver-related morbidity and mortality.

The viral mechanisms that promote the establishment of persistent HCV infection remain poorly defined. In general, infected hosts mount a variety of innate and adaptive immune responses to ongoing replication of RNA viruses. Many viruses have evolved mechanisms to evade or defeat these cellular responses (reviewed in Ref. 5), and HCV is no different. Recent studies have shown that HCV infection blocks viral activation of interferon regulatory factor 3 (IRF-3), a transcription factor that plays a pivotal role in orchestrating innate intracellular antiviral responses mediated through both the retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3) double-stranded RNA signaling pathways. HCV accomplishes this by targeting the RIG-I and TLR3 adaptor proteins, MAVS and TRIF, for specific proteolysis by the viral NS3/4A protease (6–8). It is possible that the ability of HCV to delay or prevent early innate immune responses may contribute to the establishment of persistent infection.

Subgenomic HCV RNA replicons capable of autonomous replication in stably selected cell lines were first reported by Lohmann et al. (9) in 1999. These replicon RNAs are bicistronic constructs containing the native 5'- and 3'-nontranslated RNA sequences of HCV, with the internal ribosome entry site (IRES) within the HCV 5'-nontranslated RNA driving translation of the neomycin phosphotransferase gene, followed by a heterologous IRES from encephalomyocarditis virus driving translation of the nonstructural (NS) HCV proteins (NS2 or NS3 through NS5B). These RNAs replicate autonomously when transfected into Huh7 human hepatoma cells and allow the selection of stable replicon cell lines with G418 (9). In addition, several groups have recently reported HCV cDNA clones that

mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; siRNA, small interfering RNA.
produce virus particles that are capable of undergoing a complete viral replication cycle within cultured human hepatoma cells (10–13). These replicon cell lines and newly reported virus clones represent useful tools for investigation of HCV replication and discovery of anti-HCV compounds.

Previous studies have shown that cytokines, including type I and II interferons (IFNs), interleukin-1β, and transforming growth factor-β (14–17), small molecule inhibitors of NS3 or NS5B (18, 19), and small interfering RNAs (siRNAs) specific for HCV RNA (20), can modulate the efficiency of HCV replication, reducing and in some cases even eliminating replicon RNA. The activities of a number of intracellular signaling molecules and pathways also have been shown to influence HCV replication and RNA abundance in replicon cell lines, including ERK (21), phosphatidylinositol 3-kinase (PI3K) (22), IRF-3 (6), Smad (17), and the JAK/STAT pathway (14, 15). Type I IFN-induced JAK-STAT activation results in strong suppression of viral replication by inducing a series of IFN-stimulated genes (ISGs). Although the JAK/STAT pathway has been relatively well characterized, as has the virus-activated RIG-I/IFR-3 pathway that leads to induction of type I IFN synthesis, many details of the mechanisms by which other pathways regulate HCV replication remain unclear. The state of the cell cycle and rate of cellular proliferation has also been shown to affect replication (23, 24).

The p21-activated kinases (PAKs) are evolutionarily conserved serine/threonine kinases that play an important role in a variety of cellular functions in mammalian cells, including control of cell proliferation, differentiation, migration, and cell death (reviewed in Ref. 25). PAK1, a member of the PAK family, is activated by several growth factors, including epidermal growth factor, platelet-derived growth factor, and hepatocyte growth factor via activation of the Rho GTPases, Rac1 and Cdc42 (26). The binding of GTP-bound GTPases to PAK1 dissociates dimeric PAK1 molecules and leads to the active state conformation. PAK1 is also activated by numerous physiological signals (25). It has been suggested that PAK1 mediates virus- or poly(I:C)-stimulated signaling and that it may contribute to the activation of IRF-3 through TANK-binding kinase-1 and IκB kinase-ε (27). Although further study is required, these observations suggest that PAK1 may play an important role in activating innate intracellular antiviral signaling pathways.

Here, we demonstrate that PAK1 knockdown leads to an increase in HCV replicon RNA abundance in stable replicon cell lines and enhances replication of a cell culture-infectious virus, suggesting that PAK1 is involved in cellular processes that negatively regulate HCV replication. Contrary to previous studies (27), however, we do not find PAK1 to be required for IRF-3 activation by the virus-activated RIG-I pathway. We show that PAK1 activation in these replicon cells is inhibited by rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, and that mTOR is independently regulated by both PI3K/Akt and ERK. Further analysis revealed that p70 S6 kinase is responsible for the PAK1-mediated suppression of HCV replication, suggesting a novel pathway toward PAK1 activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human hepatocellular carcinoma cell line, Huh7, was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, glucose (4.5 mg/ml) at 37 °C in a 5% CO2 atmosphere. A7 and HP, two Huh7 clonal cell lines that harbor Con1 HCV (genotype 1b) sub-genomic replicon RNAs (6), and a genome-length (HCV-N strain) replicon cell line, 2-3 (28), were maintained in complete Dulbecco’s modified Eagle’s medium with 250 µg/ml G418.

**Antibodies and Kinase Inhibitors**—Antibodies to PAK1, phospho-PAK1 (Thr-423), p70 S6 kinase, phospho-p70 S6 kinase, Akt, phospho-Akt (Ser-473) were purchased from Cell Signaling Technology. Antibodies to β-actin (A-5441) and FLAG (F-3165) were from Sigma. A mouse monoclonal antibody to the HCV core protein (C7-50) was obtained from Affinity BioReagents. A rabbit polyclonal antibody to HCV NS5A was kindly provided by Dr. Craig Cameron. The inhibitors, LY294002 (PI3K), wortmannin (PI3K), rapamycin (mTOR), and PD98059 (ERK) were obtained from Calbiochem.

**Plasmids and DNA Transfection**—pIFN-β-Luc was provided by Dr. Rongtuan Lin. pPRDII-Luc (29) and pISG56-Luc were provided by Dr. Michael Gale. pRC22F (30) has been previously described. An expression vector for eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) was constructed by amplifying specific cDNA using conventional reverse transcription-PCR methods and cloning the amplified sequences into the HindIII-EcoRI site of pFLAG-CMV-6a (Sigma). Thr-46 was mutated to Ala by site-specific mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) to construct the constitutive active mutant, pCMV-4EBP1-T46A. Cells were transfected with plasmid DNAs using TransIT-LT1 (Mirus) according to the manufacturer’s recommended procedures. Cells were co-transfected with pCMV-β-gal (Clontech) to monitor transfection efficiencies.

**RNA Interference**—Transfections of siRNA specific for PAK1 or p70 S6 kinase were carried out using Oligofectamine (Invitrogen) with a final concentration of siRNA of 100 nM, according to the manufacturer’s recommended procedures. Briefly, for transfection of cells in 24-well cell culture plates, 60 pmol of siRNA was diluted in 50 µl of Opti-MEM (Invitrogen), and 3 µl of Oligofectamine was diluted in 12 µl of Opti-MEM. After incubation for 5 min, the siRNA and Oligofectamine solutions were mixed, incubated for an additional 20 min, and added to cell culture medium. siRNAs specific for PAK1 were purchased from Dharmacon (M-003521-03); these were transfected singly or as a pool consisting of four oligoribonucleotide duplexes with the following sequences: 5′-ACCCAAACAUUGGAAUUUACU-3′ and 5′-UAUUUCACAGUUUGG- UU-3′ (P1), 5′-GGAAUUUACCAAGCAUAUU-3′ and 5′-UAUGCUUCGAUUUCUUCC-3′ (P2), 5′-UCAAAU- AACGCCCAUGACAU-3′ and 5′-UGUCAUACGGCUGUA- UUGAUU-3′ (P3), and 5′-CAUAAUUCACAUAGCU- CUU-3′ and 5′-GACUUGAUUUUGAAGU-3′ (P4). Validated negative control siRNAs (C1 and C2) with limited sequence identity to known genes were obtained from Ambion (catalog number 4611 and 4613, respectively). p70 S6 kinase-
specific siRNA (catalog number 1454) and a mutated p70 S6 kinase siRNA (MutS6K), containing two nucleotide substitutions, were purchased from Ambion as oligoribonucleotide duplexes with the sequences 5'-GGACAUUGCAGGAGUGU-UUdTdT-3' and 5'-AAACACUCCUGCAUCCGdTdTc-3' (p70 S6 kinase-specific, S6K) and 5'-GGACAUUGCAGGAGGAGGUGUUUdTdT-3' and 5'-AAACACUCCGACCAUCCGdT-c3' (MutS6K).

Promoter Reporter Assays—A7 and HP cells (5 × 10^5 cells/well in 24-well plates) were transfected with siRNA specific for PAK1 as described above. Two days later, cells were cotransfected with 350 ng of reporter plasmids and 50 ng of pCMV-β-gal DNA. Cells were lysed and assayed for luciferase activity and β-galactosidase activity 24 h later. To assess the role of PAK1 in IRF-3 activation induced by Sendai virus, Huh7 cells (5 × 10^4 cells per well in 24-well plates) were transfected with PAK1 siRNA, followed by the transfection of reporter plasmids (350 ng) and pCMV-β-gal (50 ng) 24 h later. The next day, cells were mock-treated or challenged with 100 hemagglutinin units/ml Sendai virus (Charles River Laboratory) as previously described (6) and then harvested and assayed for reporter protein activity 16 h later. The luciferase activity was normalized for transfection efficiency based on the results of the β-galactosidase assay. Assays were performed in triplicate; S.D. values are denoted by bars in the figures.

Cell Proliferation Assay—Cell proliferation was assessed by a WST-1 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) assay according to the manufacturer’s suggested protocol (Chemicon International Inc.). A7 cells (1 × 10^5 cells/well) were seeded into 96-well flat-bottom plates, transfected with negative control or PAK1 siRNA as above, and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Ten microliters of WST-1 reagent was added to each well, the cells were incubated at 37 °C for 1 h, and absorbance at 450 nm was measured using a spectrophotometer. Assays were carried out in triplicate; S.D. values are denoted by bars in the figures.

Northern Blot Analysis—For Northern blot analysis, total cellular RNA was extracted using TRIzol reagent (Invitrogen). Eight micrograms of total RNA was electrophoresed and transferred to a nylon membrane (Hybond-N; Amersham Biosciences). The membrane was probed with in vitro transcribed negative-strand HCV RNA labeled with digoxigenin, using reagents provided with the DIG Northern Starter Kit (Roche Applied Science) and following the manufacturer’s recommended procedures. After extensive washing and blocking, the membrane was hybridized with anti-digoxigenin-AP, incubated with CSPD, and exposed to a BioMax MR x-ray film (Eastman Kodak Co.).

Immunoblot Analysis—Total cellular proteins were extracted with lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 50 mM NaF, and 50 µg/ml aprotinin in phosphate-buffered saline. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences). After blocking, the membrane was probed with specific primary antibodies (see above), followed by further incubation with a secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences). Proteins were visualized using ECL Western blot detection reagents (Amersham Biosciences) and exposure to film.

Dual Luciferase Assay—Huh7 cells (5 × 10^5 cells/well) were seeded into 24-well plates and cotransfected with 200 ng of pRC22F and 200 ng of pCMV-4EBP1-T46A, as described above. Twenty-four hours later, cells were lysed and assayed for HCV IRES-dependent firefly luciferase activity and cap-dependent Renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega). For experiments involving RNA interference, Huh7 cells (5 × 10^5 cells/well) in 24-well plates were sequentially transfected with 400 ng of pRC22F and with negative control or PAK1 siRNA. After incubation for 3 days, cells were harvested and assayed for luciferase activities. Assays were performed in triplicate; S.D. values are denoted by bars in the figures.

Cell Culture-infectious HCV—H-NS2/NS3-J/YH/QL is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH1 virus and containing compensatory mutations in E1 (Y361H) and NS3 (Q1251L) (Fig. 5A) (31). These two mutations render the chimeric RNA highly infectious (31). Virus stock (10^7 focus-forming units/ml) was prepared at the fourth virus passage by clarifying supernatant media harvested from infected cells (collected after overnight incubation of the cells in Dulbecco's modified Eagle's medium without serum) by centrifugation at 3000 rpm for 5 min and then concentrating 50× in a Centrifuge Plus-80 Centrifugal Filter device with Ultracel PL Membranes (100-kDa exclusion) (Millipore Corp.).

Infections were carried out at a multiplicity of infection of ∼2. Huh7 cells (2 × 10^5 cells/well) were seeded into 6-well plates and 24 h later transfected with either PAK1-specific or control siRNA as above. Following 24 h of additional incubation, the medium was replaced with 1 ml of medium containing 4 × 10^4 focus-forming units of virus. After a 12-h incubation at 37 °C in a 5% CO2 environment, the cells were washed with phosphate-buffered saline and refed with normal culture medium. At 24, 48, and 72 h after inoculation of virus, cells were lysed and assayed for HCV core protein expression as well as PAK1 and β-actin by immunoblot. Supernatant cell culture fluids collected at 48 and 72 h were also assayed for infectious virus.

HCV Infectivity Assay—Huh7.5 cells (2 × 10^5 cells/well) were seeded onto 8-well chamber slides (Nalge Nunc) 24 h prior to inoculation of 100 µl of culture medium containing virus. After 3 days of incubation, cells were fixed in methanol/acetone (1:1) for 10 min, incubated for 2 h with anti-core antibody diluted 1:300, and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Southern Biotech) for 1 h. Nuclei were visualized with 4',6-diamidino-2-phenylindole-dihydrochloride. The cells were examined with a Zeiss LSM 510 laser-scanning confocal microscope. Clusters of infected cells staining for core antigen were counted, and virus titers were calculated in terms of focus-forming units/ml (11, 31).
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RESULTS

PAK1 Knockdown Enhances HCV Replicon RNA Abundance—To determine whether PAK1 plays a role in controlling HCV RNA replication and the abundance of viral RNA in replicon cell lines, we transfected PAK1-specific siRNA into two cell lines, A7 and HP, which contain subgenomic HCV RNA replicas constructed from the Con1 HCV sequence. As described previously (6), these cell lines have contrasting characteristics with respect to replicon abundance and basal IRF-3 activity; HP demonstrated robust RNA replication leading to a complete block in Sendai virus-induced IRF-3 phosphorylation and nuclear translocation, whereas the abundance of replicon RNA was lower in A7 cells, which displayed basal activation of IRF-3 and partial IRF-3 responses to Sendai virus infection (6). As shown in Fig. 1, siRNA-mediated knockdown of PAK1 enhanced the abundance of HCV replicon RNA as well as the level of viral NS5A protein expression. Although the starting abundances of HCV RNA and NS5A were greater in HP cells than in A7 cells, knockdown of PAK1 expression by transfection of a pool of PAK1-specific siRNAs reproducibly and significantly enhanced viral RNA and protein abundance in both replicon cell lines (Fig. 1A and B). To confirm that the enhanced replication of HCV RNA was specifically related to PAK1 knockdown and not a result of an off target effect of one of the siRNAs included in the pool, we transfected A7 replicon cells with each of the four individual PAK1-specific siRNAs as well as two negative control siRNAs comprising sequences unrelated to any known genes. As shown in Fig. 1D, all four PAK1-specific siRNAs decreased PAK1 expression levels and increased NS5A protein abundance in replicate experiments. Since these four PAK1-specific siRNAs have different sequences and target different regions of the PAK1 gene, these data argue strongly for the PAK1-specific nature of the enhancement in HCV RNA replication. We conclude from these results that PAK1 suppresses HCV RNA and protein abundance.

PAK1 Control of HCV Replication Is Independent of RIG-I and IRF-3—IRF-3 plays a pivotal role in orchestrating innate intracellular antiviral defenses that may control or eliminate virus infections. Previous experimental results demonstrated that IRF-3 is basally activated in A7 cells and that antiviral genes regulated by IRF-3, such as ISG15 and ISG56, are expressed at low levels in these cells (6). In addition, the ectopic expression of a dominant negative IRF-3 caused an up-regulation of HCV abundance in these cells (6). Since PAK1 has been suggested to contribute to IRF-3 activation upstream of the TANK-binding kinase-1 and IκB kinase-ε kinases (27), it is possible that the enhancement of HCV replication in A7 cells could be due to inhibition of basal IRF-3 activity. On the other hand, the increase in HCV RNA following PAK1 knockdown in HP cells (Fig. 1) cannot be explained by such a mechanism, since IRF-3 activity is completely blocked in these cells under basal conditions (6). Thus, taken together, the data shown in Fig. 1 suggest that PAK1 suppresses HCV replication through an IRF-3-independent mechanism.

To formally demonstrate this, we first determined whether PAK1 is required for virus activation of IRF-3 in these replicon cells, since this has been suggested to be the case in other cell
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**FIGURE 2.** PAK1 knockdown does not impair activation of IRF-3 through the RIG-I pathway. A, Huh7 cells were harvested before and at the indicated time point after infection with Sendai virus. Total and phosphorylated PAK1 were detected by immunoblot analysis. B, PAK1 knockdown reduces NF-κB but not IRF-3-responsive promoter activities in Huh7 cells. Cells grown in 24-well plates were transfected with negative control siRNA C1 or C2 or PAK1-specific siRNA followed by cotransfection with pCMV-β-gal and pISG56-Luc (top), pIFN-β-Luc (middle), or pPRDII-Luc (bottom). Cells were mock-treated or infected with Sendai virus (100 hemagglutination units/ml) 16 h prior to harvest for luciferase assay. The bars show the average relative luciferase activity ± S.D. in triplicate cultures. C, influence of PAK1 knockdown on IRF-3- and NF-κB-responsive promoters in HCV replicon cells. A7 and HP cells grown in 24-well plates were transfected with negative control siRNA C1 or C2 or PAK1-specific siRNA. Two days later, cells were cotransfected with pCMV-β-gal and pISG56-Luc (top), pIFN-β-Luc (middle), or pPRDII-Luc (bottom) and subsequently assayed for luciferase activity.

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**FIGURE 3.** Cell proliferation is decreased by PAK1 knockdown. A, A7 cells were plated into 96-well plates and transfected with PAK1 siRNA or negative control siRNAs C1 and C2. Cells were incubated for the days indicated, and cell viability was assessed using the WST assay. B, immunoblot analysis of PAK1 and β-actin abundance in A7 cells transfected with PAK1-specific siRNA or negative control siRNAs, C1 and C2.

down of PAK1 in Huh7 cells. As shown in Fig. 2B, PAK1 knockdown did not affect Sendai virus activation of either the ISG56 or IFN-β promoters. Finally, we assessed the impact of PAK1 knockdown directly on basal IRF-3-responsive promoter activity in A7 and HP cells, in the absence of Sendai virus infection. Although we could not demonstrate the difference in the basal activities of IRF-3-responsive promoters reported previously in these cells (6) (possibly because additional cell culture-adaptive mutations may have occurred in the replicon RNAs during continued passage of the cell lines), these results demonstrated that PAK1 knockdown does not suppress basal activities of the ISG56 or IFN-β promoters in either cell line (Fig. 2C). We conclude from these results that PAK1 is not involved in the regulation of IRF-3 activity in Huh7 cells and that the enhanced abundance of HCV RNA that follows PAK1 knockdown is not due to suppression of IRF-3 activity.

On the other hand, PAK1 knockdown moderately reduced Sendai virus-induced activation of the PRDII promoter, an NF-κB-responsive component of the IFN-β enhancerosome (Fig. 2B). The basa activity of this promoter was also suppressed by PAK1 knockdown, particularly in HP cells (Fig. 2C). This is consistent with a previous report indicating that PAK1 may regulate NF-κB activity (35).
Although transfection of two different negative control siRNAs resulted in no apparent change in the growth of the cells, a significant decrease in cellular proliferation was observed in cells transfected with PAK1 siRNA (Fig. 3A). This decrease in cellular proliferation was associated with a slight reduction in the PAK1 expression level 24 h post-transfection of PAK1-specific siRNA with substantially greater suppression evident at 48–72 h (Fig. 3B). Similar results were obtained in HP cells (data not shown). These results indicate that the up-regulation of HCV abundance following PAK1 knockdown is not due to pleiotropic effects promoting cellular proliferation. Indeed, since viral RNA replication has been shown to be substantially reduced in slowly growing cells (23, 24), the fact that PAK1 knockdown enhances viral RNA abundance while significantly slowing cell proliferation suggests that PAK1 may have a greater effect on HCV RNA replication than suggested simply by the magnitude of the increase in viral RNA abundance. These competing effects of PAK1 on cell proliferation and viral replication may account for the varying increases in NS5A abundance in relation to the magnitude of PAK1 knockdown that we observed following transfection of the individual siRNAs in Fig. 1C.

PAK1 Is Basally Activated in Replicon Cells—Next, we asked whether PAK1 is basally activated in the replicon cells, by comparing the abundance of PAK1 phospho-Thr-423 in the A7 and HP replicon cells with that in Huh7 cells. We found the abundance of phosphorylated PAK1 to be significantly greater in the A7 and HP cells (Fig. 4A). This suggested two possibilities: first, that the presence of the HCV replicon specifically induces phosphorylation of PAK1 or, alternatively, that cells with a high level of PAK1 phosphorylation were selectively isolated during the G418 selection of the replicon cell lines. To distinguish between these possibilities, we “cured” the A7 and HP cell lines, eliminating HCV replicon RNAs by IFN-α treatment (200 units/ml) over a period of 2 weeks. Elimination of the HCV replicon was confirmed by the absence of NS5A expression in the cured cells (Fig. 4B). Following passage of the cells in the absence of IFN-α, we again determined the abundance of phosphorylated PAK1. As shown in Fig. 4B, there was no significant difference in the abundance of phosphorylated PAK1 in A7 and HP cells before and after elimination of the replicon RNA. We also examined PAK1 activation in a third HCV replicon cell line, 2-3, which contains a dicistronic RNA replicon encoding the entire HCV polyprotein (28). The phosphorylation levels of PAK1 in 2-3 or 2-3c cells (their interferon-α-cured progeny) were also greater than in normal Huh7 cells (Fig. 4C). These results indicate that HCV RNA replication does not induce PAK1 phosphorylation in Huh7 cells. Rather, they suggest the surprising conclusion that cells possessing a higher level of phosphorylated PAK1 may be preferentially selected during the clonal establishment of HCV replicon cell lines, despite the fact that PAK1 appears to negatively regulate HCV RNA replication and both viral RNA and protein abundance (Fig. 1).

Replication of Cell Culture-infectious HCV Is Enhanced by PAK1 Knockdown—Although PAK1 knockdown enhanced viral RNA and protein abundance in stable replicon cell lines as described above, it was of interest to confirm these effects on replication of virus in a cell culture model of HCV infection. To accomplish this, we utilized H-NS2/NS3-J/YH/QL virus, a genotype 1a (H77-S) 2a (JFH-1) chimera (Fig. 5A) that is highly infectious in cultured Huh7 cells (31). We transfected Huh7 cells with PAK1-specific or control siRNAs prior to inoculation with virus at a high multiplicity of infection (≈2) and then monitored viral protein abundance in the cells as well as the yield of infectious virus released into the media. As shown in Fig. 5B, a positive effect of PAK1 knockdown on HCV core protein expression was evident as early as 24 h postinfection (Fig. 5B, compare lanes 1 and 2). Core protein abundance was also increased later in the infectious cycle, 2–3 days postinoculation, when core protein expression was robust (compare lanes 5 and 6). This regulation of HCV infection by PAK1 knockdown was consistent with the earlier results obtained in replicon cells (Fig. 1), and confirms that PAK1 controls replication of replicons and infectious virus in a similar fashion. We also assayed infectious virus released into the media by virus-infected cells in the presence and absence of PAK1 knockdown (Fig. 5C). Approximately 2-fold higher titers of infectious virus were present in
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FIGURE 5. PAK1 knockdown enhances the replication of cell culture-infectious virus in Huh7 cells. A, organization of the genotype 1a/2a H-NS2/NS3-J/YH/QL chimeric HCV genome, which carries compensatory mutations in E1 (Y361H) and NS3 (Q1251L) that promote its efficient replication in cultured cells. The shaded region of the polyprotein coding segment is derived from H77 virus, whereas the remainder of the genome is from JFH1. B, immunoblot analysis showing that PAK1 knockdown enhances expression of core protein in HCV-infected Huh7 cells. Cells were treated with negative control siRNA (C) or siRNA specific for PAK1, prior to inoculation with infectious virus at a multiplicity of infection of 2. Cells were harvested 1, 2, and 3 days post-inoculation, and cell lysates were assayed for core, PAK1, and β-actin expression. Immunoblot analyses for core were carried using 1 or 5 μg of total protein resolved on each lane. C, virus yield is enhanced by PAK1 knockdown in Huh7 cells inoculated with cell culture-infectious virus. The titer of infectious virus was determined by fluorescent foci counting in supernatant media collected 2 and 3 days after inoculation of cells with virus. Bars, mean virus yield (focus-forming units/ml) ± S.D. in four independent experiments. * difference is significant at \( p < .05 \).

FIGURE 6. Inhibitors of PI3K (LY294002) or mTOR (rapamycin) suppress phosphorylation of PAK1 and p70 S6 kinase and cause an increase in HCV replicon RNA and viral protein abundance. A and HP cells were treated with 20 μM LY294002 (A and B) or 100 nM rapamycin (C and D) for 24 h or left untreated. A and C, immunoblots showing the abundance of total and phosphorylated p70 S6 kinase, total and phosphorylated PAK1, NS5A, and β-actin following treatment of the A7 and HP cells with the chemicals inhibitors. B and D, Northern blot analysis of HCV RNA following treatment of A7 and HP cells with the inhibitor compounds.

supernatant fluids from cells subjected to PAK1 knockdown, which is consistent with the observed increase in core protein abundance.

LY294002 and Rapamycin Regulate PAK1 Activity and HCV Replication—PAKs are regulated by a variety of signals, including the activation of Rho family GTases, PI3K, and membrane localization (25). Among these, the PI3K pathway appears to be particularly important, since it plays a major role in transducing many stimuli. To determine how PAK1 phosphorylation is regulated in the A7 and HP cells, we examined the activity of PAK1 following treatment of the cells with various protein kinase inhibitors. We found that LY294002, an inhibitor of PI3K, almost completely suppressed the phosphorylation of PAK1 when added to cell culture media at 20 μM for 24 h (Fig. 6A). Consistent with the observed reduction in PAK1 phospho-Thr-423, treatment of the replicon cells with LY294002 enhanced expression of the viral NS5A protein (Fig. 6A) and resulted in an increased abundance of viral RNA (Fig. 6B).

Phosphorylation of the p70 S6 kinase was also inhibited in cells treated with LY294002 (Fig. 6A), which is consistent with its regulation by mTOR, which is known in turn to be activated downstream of PI3K (40). This suggests that LY294002 inhibits mTOR in the replicon cells, presumably through its action on PI3K, raising the possibility that PAK1 regulation of HCV replication occurs through a PI3K-mTOR pathway. To investigate this possibility, we treated the replicon cells with 100 nM rapamycin, an mTOR inhibitor. Strikingly, rapamycin treatment completely abrogated phosphorylation of p70 S6 kinase and PAK1 (Fig. 6C). In addition, as expected, rapamycin treatment led to increases in the abundance of HCV NS5A (Fig. 6C) and replicon RNA (Fig. 6D). However, the enhanced expression of NS5A in rapamycin-treated cells differed both qualitatively and quantitatively from the changes observed after treatment with LY294002. First, the increase in NS5A abundance was somewhat less following rapamycin compared with LY294002 treatment of the cells. More importantly, the 58-kDa hyperphosphorylated species of NS5A was selectively increased in LY294002-treated compared with rapamycin-treated cells (Fig. 6C, compare A and C). These results suggest that another signaling partner downstream of PI3K, in addition to mTOR, may influence both HCV replication and the status of NS5A hyperphosphorylation.

The exact role of NS5A in the replication of HCV RNA is not clear. However, several previous reports suggest that the phosphorylation state of NS5A is intimately linked with the efficiency of HCV RNA replication. Decreased hyperphosphorylation of NS5A may stimulate HCV RNA replication, possibly by enhancing the interaction of NS5A with the host protein hVAP-A (41–43). Recent data suggest that CK1 kinase is responsible for the hyperphosphorylation of NS5A (44). It is interesting that LY294002 treatment of replicon cells enhanced viral RNA replication while increasing the abundance of the hyperphosphorylated NS5A species. Further studies are needed to understand the significance of the difference in NS5A p58
expression, but, taken collectively, these data indicate that PAK1 suppression of HCV replication is regulated, at least in part, by mTOR, and upstream of mTOR by PI3K.

FIGURE 7. p70 S6 kinase knockdown inhibits PAK1 phosphorylation and enhances HCV protein abundance. A, immunoblots showing the impact of siRNA knockdown of S6 kinase on the abundance of total and phosphorilated PAK1, NS5A, and β-actin. A7 and HP cells were transfected with negative control siRNA, C1 or C2, S6 kinase-specific siRNA (S6K), or mutant S6 kinase siRNA (MutS6K (Mut)). Three days later, cells were harvested, and protein abundance was determined by immunoblot analysis. B, ectopic expression of 4EBP1-T46A enhances HCV protein abundance in replicon cell lines. A7 and HP cells were transfected with empty vector or the 4EBP1-T46A expression vector. Immunoblot analyses were carried out to determine the abundance of total and phosphorylated PAK1, NS5A, 4EBP1-T46A (using anti-FLAG antibody), and β-actin. C, 4EBP1-T46A expression also enhances HCV RNA abundance in replicon cell lines. Northern blot analysis for HCV RNA using total RNA extracted from A7 and HP cells transfected with empty vector or 4EBP1-T46A expression vector. D, schematic showing the organization of dicistronic reporter plasmids containing the Renilla luciferase and firefly luciferase sequences. E, impact of 4EBP1-T46A expression and PAK1 knockdown on HCV IRES-specific translation. Left, Huh7 cells were cotransfected with pRC22F and pCMV-4EBP1-T46A or empty vector. 24 h later, cells were harvested and assayed for HCV IRES-dependent firefly luciferase activity (empty bars) and cap-dependent Renilla luciferase activity (shaded bars). Right, Huh7 cells were transfected with pRC22F followed by treatment with negative control siRNA C1 or C2 or PAK1-specific siRNA. Three days later, cells were harvested and assayed for luciferase activities. The activity of each luciferase was calculated as the percentage of that present in lysates of cells transfected with empty vector and C1 control siRNA.
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was enhanced by the expression of 4EBP1-T46A (Fig. 7, B and C). Thus, both substrates of mTOR, p70 S6 kinase and 4EBP1, appear capable of independently regulating the abundance of HCV proteins in the replicon cells. However, only p70 S6 kinase regulates the activation of PAK1, suggesting an alternative mechanism for NS5A regulation by 4EBP1.

Since 4EBP1 suppresses cap-dependent translation, we considered the possibility that it might positively regulate HCV protein expression and/or replication by stimulating translation of the viral polyprotein, which is initiated in a cap-independent process mediated by the encephalomyocarditis virus IRES placed between the two cistrons of the replicon (9). To test this hypothesis, we co-transfected replicon cells with the 4EBP1-T46A expression vector and pRC22F, a dicistronic reporter vector expressing Renilla luciferase by cap-dependent translation, and a downstream firefly luciferase coding sequence under cap-dependent control of the HCV IRES (Fig. 7D). As expected, cap-dependent translation was reduced by the ectopic expression of 4EBP1-T46A (about 40%), whereas IRES-directed translation of the firefly luciferase was increased about 2.5-fold (therefore leading to a 5-fold increase in viral translation relative to cellular cap-dependent translation (Fig. 7E, left). This suggests that 4EBP1 expression might favorably influence HCV replication by stimulating viral translation, independent of any effect on PAK1 activation. This is consistent with the results we observed in the experiment shown in Fig. 7, B and C.

We also assessed cap-dependent versus IRES-directed translation following siRNA knockdown of PAK1. Under these conditions, HCV IRES activity was not enhanced, and cap-dependent translation was only slightly decreased (about 30%) (Fig. 7E, right). These results suggest that PAK1 is unlikely to regulate HCV replication by directly modulating the activity of the HCV IRES.

PI3K and ERK Pathways Lead to Activation of p70 S6 Kinase—To confirm that the LY294002-dependent suppression of mTOR/p70 S6 kinase (Fig. 6A) was due to suppression of PI3K activity, we determined the status of Akt phosphorylation following treatment of the cells with wortmannin, another inhibitor of PI3K, as well as LY294002. Akt is an intermediate in the PI3K-mTOR pathway (40). A7 and HP cells were treated with these inhibitors for 1 h prior to lysis and immunoblot analysis. Surprisingly, LY294002 and wortmannin had opposing effects on the phosphorylation of Akt and p70 S6 kinase under these conditions (Fig. 8A). LY294002 inhibited p70 S6 kinase phosphorylation completely and weakly inhibited Akt phosphorylation. In contrast, wortmannin inhibited Akt phosphorylation completely while only partially suppressing p70 S6 kinase phosphorylation (Fig. 8A). These effects were similar in both the A7 and HP replicon cell lines. On the other hand, inhibition of mTOR by rapamycin strongly enhanced Akt phosphorylation, suggesting that there is feedback regulation of Akt by mTOR. Since siRNA knockdown of p70 S6 kinase did not influence the phosphorylation status of Akt (data not shown), this feedback regulation does not appear to be through p70 S6 kinase. The brief period of inhibitor treatment in these experiments did not allow for observations of the effect of inhibitor treatment on HCV replication.

We further investigated the kinetics of Akt phosphorylation in response to LY294002 (Fig. 8B). Both the A7 and HP cell lines showed rapid loss of Akt phosphorylation following the addition of LY294002 to cell culture media. This early response is consistent with LY294002 inhibition of the canonical PI3K pathway, which leads to Akt activation in normal cells. After 1 h, Akt phosphorylation gradually increased. However, this late increase in Akt phosphorylation was inhibited by wortmannin (Fig. 8B, compare lanes 5 and 7). This suggests the possibil-
ity of negative feedback in the regulation of PI3K, with the rephosphorylation of Akt involving a wortmannin-sensitive but LY294002-insensitive PI3K-related kinase. However, since wortmannin blocked Akt activation completely, the partial inhibition of p70 S6 kinase by wortmannin implies that p70 S6 kinase may be regulated by other means in addition to the PI3K/Akt pathway. On the other hand, LY294002 inhibited p70 S6 kinase phosphorylation completely, even after rephosphorylation of Akt. This raises the possibility that LY294002 may inhibit the pathway at some point downstream of Akt, perhaps mTOR itself, as a previous report has suggested (48).

Finally, we observed that inhibition of ERK by PD98059 partially suppressed p70 S6 kinase (Fig. 8C). More importantly, a combination of PD98059 and wortmannin completely abolished the phosphorylation of p70 S6 kinase, suggesting that PI3K and ERK are both likely to regulate mTOR activation, PAK1 phosphorylation, and thus cellular control of HCV RNA replication.

**DISCUSSION**

We have shown here that HCV replication is suppressed by activation of PAK1. Although our studies were initiated in response to a report that PAK1 may act upstream of TANK-binding kinase-1 and Ikk kinase-ε in signaling pathways leading to activation of IRF-3 (27), the PAK1 regulation of HCV replication that we have demonstrated here appears to occur independently of IRF-3 activation. Virus activation of IRF-3 and attendant type I interferon responses is known to occur through several distinct pathways involving different pathogen-associated molecular pattern receptors. TLR3 and its adaptor TRIF (49) initiate signaling on binding extracellular double-stranded RNA, whereas the RIG-I and its homolog MDA5 recognize viral RNAs within the cytoplasm and signal to IRF-3 through the adaptor protein, MAVS (8). The two HCV replicon cell lines we used in these studies, A7 and HP, are derived from Huh7 cells and thus express negligible amounts of TLR3 (32). Furthermore, the RIG-I/MAVS pathway is completely disrupted in HP cells due to the expression of a high abundance of TLR3 and MAVS (8), whereas the RIG-I and its homolog MDA5 recognize viral RNAs within the cytoplasm and signal to IRF-3 through the adaptor protein, MAVS (8). The two HCV replicon cell lines we used in these studies, A7 and HP, are derived from Huh7 cells and thus express negligible amounts of TLR3 (32). Furthermore, the RIG-I/MAVS pathway is completely disrupted in HP cells due to the expression of a high abundance of the HCV NS3 protease, which efficiently targets MAVS for proteolysis (50). Since neither the TLR3 or RIG-I pathway is active in HP cells, it is difficult to attribute the increase in HCV replicon abundance observed with PAK1 knockdown (Fig. 1) in HP cells to inhibition of an IRF-3-mediated response.

However, we did assess the possibility that PAK1 could play an essential role in the RIG-I activation of IRF-3 in Huh7 cells by determining whether siRNA knockdown of PAK1 interfered with IRF-3 activation by Sendai virus, a potent stimulator of the RIG-I pathway. We found no differences in the activation of IRF-3-responsive promoters following Sendai virus infection of Huh7 cells in the presence or absence of PAK1 knockdown (Fig. 2B). Moreover, Sendai virus infection was not accompanied by an increase in PAK1 phosphorylation (Fig. 2A). These data suggest that RIG-I-mediated activation of IRF-3 is not dependent upon PAK1 in Huh7 cells. Moreover, we also found that poly(I:C) stimulation of TLR3 did not lead to an increased abundance of phosphorylated PAK1 in PH5CH8 cells (data not shown), a hepatocyte-derived cell line that does express abundant TLR3 (32). This suggests that the TLR3 pathway is also likely to operate independently of PAK1. Our results are consistent with those reported recently by Noyce et al. (51), who demonstrated that PAK1 is not essential for IRF-3 induction of interferon-stimulated genes mediated by Sendai virus.

Importantly, we demonstrated that viral protein expression and the yield of cell culture-infected HCV were both enhanced by PAK1 knockdown in cells infected with an intergenotypic HCV chimera (Fig. 5). These data extend the range of HCV genotypes regulated by PAK1 beyond the genotype 1b replicons present in the HP and A7 cell lines, since the nonstructural protein-coding and nontranslated RNA segments of the chimeric H-NS2/NS3-J/YH/QL virus are derived from the genotype 2a JFH1 virus (31). Patients infected with genotype 1 versus genotype 2 viruses are known to respond differently to interferon treatment, with a high rate of sustained viral response in genotype 2-infected patients. Moreover, Ishii et al. (52) have demonstrated that the suppressive effect of cyclosporin on replicon RNA abundance is specific for genotype 1b. Cyclosporin has a lesser effect on JFH-1 replication, possibly because the cellular replication cofactor, cyclophilin B, which stimulates the RNA binding activity of genotype 1b NS5B, does not appear to be required for replication of JFH1 (52). These data indicate that there may be considerable diversity in the replication phenotypes of different HCV genotypes. Although further studies are needed, the results reported here suggest that PAK1 knockdown enhances both replicon RNA abundance as well as the replication of cell culture-infected virus independent of genotype.

PAK1 may be activated through multiple PI3K-dependent pathways. In the cell lines we studied, we found that PI3K and ERK both contributed to PAK1 activation. Rac1 (or Cdc42) has been shown to bind to PAK1, leading to auto- phosphorylation and activation (26). However, the pathway(s) between PI3K and Rac1/PAK1 are not well delineated. PI3K is known to activate Akt, leading to the activation of a number of cellular signaling partners and regulation of proliferation and survival/apoptosis. In the cell lines we studied, PAK1 was regulated by mTOR, which we further demonstrated to be activated by PI3K and ERK in experiments involving the use of specific inhibitors (Fig. 9) (53, 54). Rapamycin binds to mTOR and inhibits its phosphorylation, thus triggering suppression of the cap-dependent cellular protein synthesis machinery (55). Rapamycin treatment reduced PAK1 phosphorylation in replicon cells while enhancing HCV RNA replication (Fig. 6, C and D). A similar effect of mTOR on HCV replication was recently described by Mannova and Beretta (22).

The potential roles of PI3K/Akt, ERK, and the mTOR/p70 S6 kinase pathway, as defined in our studies, are depicted in Fig. 9. Our data suggest a model in which mTOR is regulated by both Akt and ERK and in which there is negative feedback between mTOR and PI3K. This feedback is not mediated by p70 S6 kinase, because the level of phosphorylated Akt did not change when p70 S6 kinase expression was disrupted by siRNA (data not shown). Although mTOR is believed to be regulated by PI3K, Ma et al. (54) demonstrated that ERK phosphorylates tuberin (TSC2) and modulates mTOR signaling. They showed
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![Diagram showing pathways by which PI3K and ERK/mTOR/p70 S6 kinase suppress HCV replication through PAK1.](image)

FIGURE 9. Schematic showing pathways by which PI3K and ERK/mTOR/p70 S6 kinase suppress HCV replication through PAK1. PAK1 is regulated by mTOR downstream of PI3K and ERK. Among the two substrates of mTOR, 4EBP1 and p70 S6 kinase, only the p70 S6 kinase activates PAK1, although phosphorylation of 4EBP1 by mTOR also suppresses HCV replication through an alternative mechanism involving down-regulation of IRES-directed translation.

that either wortmannin or U0126 (an ERK inhibitor) could suppress p70 S6 kinase phosphorylation and that these inhibitors had a synergistic effect when used in combination. Considering our results in the context of previous reports, it is possible that the relative contributions of PI3K and ERK to the activation of mTOR may vary in different cell types. In addition, the mechanism by which Akt rephosphorylation occurs following exposure to LY294002 remains unknown. We speculate that canonical (i.e. wortmannin- and LY294002-sensitive) PI3K activates Akt under normal circumstances, whereas a putative noncanonical (wortmannin-sensitive, LY294002-insensitive) PI3K is activated following LY294002 inhibition of the canonical kinase. Consistent with this interpretation, Sharrard et al. (56) have suggested that rephosphorylation of Akt is mediated by a PI3K possessing a higher than usual IC50 for LY294002. These observations would explain our results, although this PI3K is not well characterized.

mTOR has two known substrates, the p70 S6 kinase and 4EBP1, which together are responsible for mTOR-dependent regulation of cellular translation. Experiments in which we used specific siRNAs to knock down p70 S6 kinase demonstrated that this kinase is upstream of PAK1 (Fig. 7A), whereas ectopic expression of a constitutively active 4EBP1 did not influence the abundance of phosphorylated PAK1 in the HCV replicon cells (Fig. 7B). Although disruption of p70 S6 kinase abolished PAK1 phosphorylation (Fig. 7A), it is not likely that p70 S6 kinase phosphorylates PAK1 directly, because the consensus phosphorylation motif of p70 S6 kinase (K/R)XX(R/K)XX(X/S/T)X (57) is not present at sites of PAK1 phosphorylation. However, PAK1 has been recognized to form complexes with the proteins serine/threonine phosphatase 2A and p70 S6 kinase (58), suggesting that there may be an interaction between PAK1 and p70 S6 kinase.

We also found that HCV replicon abundance was enhanced by expression of constitutively active 4EBP1, which binds to eukaryotic translation initiation factor 4E and blocks translation in the same way as nonphosphorylated 4EBP1. These observations confirm a recent report by Murata et al. (21). This enhancement seems likely to be due to increased efficiency of IRES-directed translation in the context of decreased cap-dependent translation (Fig. 7E, left). In contrast, PAK1 knockdown had no effect on HCV IRES activity (Fig. 7E, right). These results thus showed that both known mTOR substrates are capable of suppressing HCV replication but that they do so through independent mechanisms. Our data indicate that PAK1 plays a primary role in the p70 S6 kinase-mediated suppression of HCV replication.

Several previous studies have investigated the relationship between HCV RNA replication and PI3K/mTOR signaling. The PI3K has been shown to be activated by a direct interaction between NS5A and the p85 regulatory subunit of PI3K as well as by the enhanced expression of N-Ras in HCV replicon cells (22). N-Ras-mediated PI3K activation resulted in mTOR activation, and when mTOR was knocked down, HCV abundance was enhanced. These earlier findings are consistent with our results, since we found HCV RNA replication to be suppressed by either of the two mTOR substrates (Fig. 7). However, although we found PAK1 to be activated in HCV replicon cells (Fig. 4, A and C), this did not appear to be due to the presence of HCV proteins, since it persisted after the replicon RNAs were eliminated from the A7, HP, and 2-3 cell lines by interferon treatment (Fig. 4, B and C). Thus, PAK1 activation is constitutive in these replicon cells lines. These results suggest that cells with high PI3K activity may have a selective advantage during the isolation of G418-resistant Huh7 cell colonies containing replicon RNA. Although this may seem contrary to the notion that PAK1 (and PI3K) may negatively regulate the replication of HCV, it may reflect other phenotypic properties of cells with high PI3K, including enhanced proliferation (Fig. 3) or cell survival.

The ability of mTOR to suppress HCV replication has been suggested to be due to the ability of the p70 S6 kinase to hyperphosphorylate NS5A (57). However, this seems unlikely, since our results indicate that PAK1, which appears to be activated by it (Fig. 9), also suppresses HCV replication. Significantly, siRNA knockdown of either kinase did not result in a reduction of the hyperphosphorylated form of NS5A in the replicon cell lines we studied (Figs. 1B and 7A).

The mechanism by which PAK1 influences HCV replication is unclear. Since PAK1 may activate members of the mitogen-activated protein kinase family (ERK, c-Jun NH2-terminal kinase, and p38) (36, 38, 39), it is possible that PAK1 regulates HCV replication through these kinases. However, treatment of the replicon cells with rapamycin did not decrease the phosphorylation of these kinases, implying that these pathways are irrelevant to PAK1-mediated regulation of HCV replication (data not shown). Similarly, inhibition of NF-κB, which is also regulated by PAK1, by a chemical inhibitor (BAY 11-7082) did not enhance but rather reduced replicon abundance (data not shown), suggesting that the
NF-κB signaling pathway is not involved either. Some cytoskeleton molecules have been demonstrated to be phosphorylated or otherwise regulated by PAK1 (25), and it is possible that they may play a role in HCV replication. Op18/stathmin normally associates with microtubules and disrupts microtubule dimerization (59). When phosphorylated by PAK1, it no longer associates with microtubules, suggesting that PAK1 contributes to the stabilization of microtubules. Since microtubules appear to be required for HCV replication (60), it is unlikely that this specific action of PAK1 suppresses HCV replication. However, the influence of PAK1 on other cytoskeleton molecules and their potential relationship to HCV replication is unclear.

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