Utilization of host SR protein kinases and RNA-splicing machinery during viral replication

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Although the viral genome is often quite small, it encodes a broad series of proteins. The virus takes advantage of the host-RNA-processing machinery to provide the alternative splicing capability necessary for the expression of this proteomic diversity. Serine–arginine-rich (SR) proteins and the kinases that activate them are central to this alternative splicing machinery. In studies reported here, we use the HIV genome as a model. We show that HIV expression decreases overall SR protein/activity. However, we also show that HIV expression is significantly increased (20-fold) when one of the SR proteins, SRp75 is phosphorylated by SR protein kinase (SRPK)2. Thus, inhibitors of SRPK2 and perhaps of functionally related kinases, such as SRPK1, could be useful antiviral agents. Here, we develop this hypothesis and show that HIV expression down-regulates SR proteins in Flp-In293 cells, resulting in only low-level HIV expression in these cells. However, increasing SRPK2 function up-regulates HIV expression. In addition, we introduce SR protein phosphorylation inhibitor 340 (SRPIN340), which preferentially inhibits SRPK1 and SRPK2 and down-regulates SRp75. Although an isonicotinamide compound, SPRIN340 (or its derivatives) remain to be optimized for better specificity and lower cytotoxicity, we show here that SRPIN340 suppresses propagation of Sindbis virus in plaque assay and variably suppresses HIV production. Thus, we show that SRPK, a well known kinase in the cellular RNA-processing machinery, is used by at least some viruses for propagation and hence suggest that SRPIN340 or its derivatives may be useful for curbing viral diseases.

HIV | kinase inhibitor | SR protein phosphorylation inhibitor 340

HIV-1 precursor RNA transcribed from proviral DNA integrated in the host cell genome contains all of the transcribed viral reading frames (1). Alternative splicing is essential for producing mRNAs encoding various viral proteins from the limited size of a single precursor mRNA (2). In the early phase of HIV expression, eight splice acceptor sites compete for the splicing machinery to produce the vif, vpu, vpr, nef, env, tat, and rev mRNAs (3). In the late phase of the virus life cycle, singly spliced longer RNA is translated to a polyprotein and then cleaved by HIV protease to generate gag and pol proteins. Several reports show that regulation of the complex splicing pattern can dramatically affect HIV-1 infectivity and pathogenesis (4–6). However, little is known about the molecular mechanism that links this alternative splicing regulation and the dynamics of virus propagation.

Alternative splicing depends on the alternative utilization of four 5′ splice sites and eight 3′ splice sites (3). The combination of these splice sites are regulated by cis-regulatory elements, which bind cellular heterogeneous nucleoproteins (hnRNPs) of the A, B, and H groups and serine–arginine-rich (SR) proteins (7). SR proteins are highly conserved in eukaryotes and are characterized by having one or two RNA-recognition motifs at the amino terminus and an arginine–serine-rich (RS) domain at the carboxyl terminus (7, 8). Extensive phosphorylation of serine residues in the RS domain occurs in all SR proteins (9, 10), which exist predominantly in a highly phosphorylated state *in vivo* (11).

The precise physiological role of this phosphorylation is still unknown. However, it is reasonable to expect that phosphorylation of SR proteins affects their protein–protein and protein–RNA interactions, intracellular localization and trafficking, and alternative splicing of precursor mRNA (12). Consistent with this idea, SR protein kinase (SRPK)-dependent herpes simplex virus splicing and SRPK-mediated phosphorylation of the hepatitis B virus core protein were reported in these viral diseases (13–15). Therefore, SR proteins and the kinases that phosphorylate them could be practical targets for therapeutic modulation of alternative splicing in diseases and viral infections where alternative mRNA splicing is important (16).

By extensively screening a chemical library for inhibitors of SRPKs, we initially found a benzothiazol compound, TG003, that has an inhibitory effect on the activity of Clk1 (17). TG003 suppresses SR protein phosphorylation, blocks dissociation of nuclear speckles, and inhibits Clk1-dependent alternative splicing in mammalian cells. Other research (18) has shown that tricyclic quinoxaline derivatives inhibit SRPK1 kinase activity but that these small molecules also inhibit a broad range of other kinases. Here, we define the relationship between RNA processing and viral replication and introduce an inhibitor of SRPK1.

We show here that (*i*) HIV infection down-regulates SR proteins in Flp-In293 cells and results in low level HIV expression; (*ii*) increasing SRp75 and the SRPK kinases that stabilize and activate SRp75 up-regulates HIV expression; (*iii*) an isonicotinamide compound, SR protein phosphorylation inhibitor (SRPIN)340, which we identified by extensively screening a chemical library, specifically inhibits SRPK and down-regulates SRp75; and (*iv*) SRPIN340 variably inhibits HIV expression but regularly inhibits expression of another RNA virus (Sindbis). Finally, we present evidence suggesting that SRPIN340 may be most effective for inhibiting acutely replicating viruses. Thus, we show that kinases involved in RNA processing are important for viral replication and propose that targeting these kinases may be a useful strategy to control viral diseases.

Results

HIV Infection Alters the Phosphorylation State and Localization of SR Proteins. The mAb 1H4 detects phosphoepitopes in the RS domain of SR proteins (19). 1H4 can be used in confocal

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Conflict of interest statement: No conflicts declared.

Abbreviations: ASF, alternate splicing factor; DIC, differential interference contrast; hnRNP, heterogeneous nucleoproteins; SF2, splicing factor 2; SR, serine–arginine-rich; RS, arginine–serine-rich; SRPK, SR protein kinase; mSRPK, mouse SRPK; SRPIN340, SR protein phosphorylation inhibitor 340.

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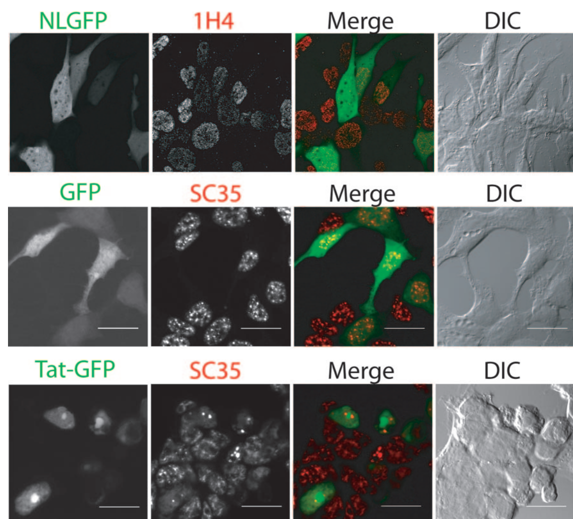

Fig. 1. HIV alters the phosphorylation of SR proteins and the localization of SC35. The first image in each row is a single image of GFP. The second image in each row is for Alexa Fluor 555. The third image is a merged image, and the fourth image is DIC microscopy. (*Top*) Prepared GFP-tagged HIV virus infected HEK293 cells, and phosphorylated SR proteins were stained with an anti-phospho-SR-specific Ab 1H4 (Zymed). HIV-1 NL4-3-infected cells expressing GFP were not stained or were weakly stained by 1H4 Ab. (*Middle* and *Bottom*) Flp-In293 were transfected with GFP-expressing (*Middle*) or GFP-Tat-expressing (*Bottom*) vector and further incubated for 72 h. Cells were fixed and stained with an anti-SC35 Ab as described in *Materials and Methods*. In GFP-expressing cells, nuclear speckle structure (multiple dots) of SC35 was observed. In GFP-Tat-expressing cells, a strong GFP signal was observed in nucleolus, and the SC35 signal was weaker in the nucleus and was seen as two dots near the nucleolus. The fluorescence images of SC35 and GFP and the DIC images were taken with a confocal microscope (Olympus FV1000). (Scale bars, 20 μm.)

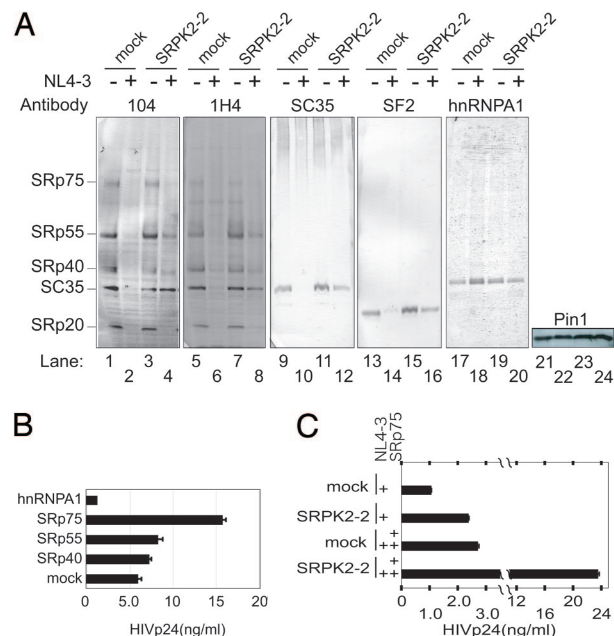

Fig. 2. HIV-1 induced dephosphorylation of SR proteins. (*A*) Western blot analysis of SR proteins in HIV-1 pNL4-3-transfected cells. Phosphorylation state of SR proteins (SRp75, SRp55, SRp40, SC35, and SRp20) and expression of SF2/ASF and SC35 were examined with anti-phospho-SR (104, lanes 1–4; 1H4, lanes 5–8), anti-SC35 (lanes 9–12), anti-SF2 (lanes 13–16), anti-hnRNPA1 (lanes 17–20), and anti-Pin1 (lanes 21–24) Abs for the whole-cell extracts prepared from the mock-overexpressing (Venus) or SRPK2-overexpressing (SRPK2-2) Flp-In293 cells with (+) or without (−) HIV-1 pNL4-3 transfection. (*B*) Effects of SR proteins on HIV-1 production. One of the expression vectors of HA-tagged SR proteins (SRp75, SRp55, and SRp40) or hnRNPA1 was introduced into the Flp-In293 cells with HIV-1 pNL4-3. After 48 h of incubation, culture supernatants were collected from each sample. (*C*) The SRPK2–SRp75 pathway enhances HIV-1 production. Culture supernatants were collected from the mock and SRPK2-2 cells at 72 h after transfection of HA-SRp75 and HIV-1 pNL4-3. To estimate the virus production level, the amount of HIV-1 p24 antigen in the culture supernatant was quantified with LUMIPULSE (Fujirebio, Tokyo, Japan) as described in ref. 28. Each sample was examined in triplicate. Values measured by LUMIPULSE are shown in the graph with standard deviations.

fluorescence microscopy studies to compare the levels of phosphorylated SR proteins in noninfected and HIV-infected cell lines. As Fig. 1 shows, phosphorylated SR proteins are readily detectable in uninfected Flp-In293 cells but decrease dramatically (almost to undetectable) when these cells are infected with HIV-1 pNL4-3 GFP, an HIV strain that carries an EGFP marker that identifies the infected cells (Fig. 1 *Top*). Results with Flp-In293 cells transfected with HIV-1 pNL4-3 are similar (data not shown), leading to the conclusion that the intracellular levels of phosphorylated SR protein detected by confocal microscopy with 1H4 decrease markedly after the introduction of the HIV genome into Flp-In293.

Western blotting analysis confirms the decrease in phosphorylated SR proteins in HIV-introduced Flp-In293 cells. As Fig. 2 shows, the phosphorylated forms of SRp75, SRp55, SRp40, SRp30, and SRp20 are clearly detectable with mAbs 104 or 1H4 in cell extracts from untransfected cells (Fig. 2*A*, lanes 1 and 5). In contrast, levels of phosphorylated SR proteins are below detectability in extracts from Flp-In293 transfected with HIV-1 pNL4-3 (Fig. 2*A*, lanes 2 and 6).

Reprobing the filter with mAbs that detect splicing factor 2 (SF2)/alternative splicing factor (ASF) and SC35, independent of phosphorylation state, surprisingly shows that the decrease in the phosphorylated forms in the HIV-transfected Flp-In293 cells is due to a loss of SR protein rather than to an inhibition of SR protein phosphorylation. Thus, as Fig. 2 shows, the levels of SF2/ASF or SC35 are dramatically decreased in HIV-transfected cell extracts (Fig. 2*A*, lanes 10 and 14). Other members of the SR protein family (SRp75, SRp55, and SRp40) are similarly decreased (data not shown). However, no significant alteration was observed in unrelated proteins (hnRNPA1 and Pin1; see lanes 17–20 and 21–24, respectively, in Fig. 2*A*). Thus, HIV infection/transfection selectively decreases the levels of SR proteins.

To investigate the mechanism of virus–host interaction underlying the HIV-mediated decrease in intracellular SR proteins, we overexpressed GFP or GFP-tagged HIV Tat (Fig. 1 *Middle* and *Bottom*). In the GFP-expressing cells, the GFP signal is found in the cytoplasm and nucleoplasm, and endogenous SC35 is observed in the nucleus with a typical speckled pattern. Overexpression of Tat-GFP, in contrast, caused decrease of SC35 protein and its mislocalization (Fig. 1 *Bottom*). Although the mechanism through which Tat causes this change is unclear (direct interaction of Tat with SR proteins or indirect physiological changes), this finding suggests that the expression of HIV Tat when the HIV-1 genome is expressed may play a critical role in the decrease of SR proteins in HIV-expressing cells.

It is important to note that the decrease in SR protein levels in the infected cells does not result in the total absence of these proteins. Indeed, these proteins are detectable at low levels by confocal microscopy and gel analysis (see Figs. 1 and 2*A*) and, as data presented here will show, can either be increased or decreased by experimental manipulations that correspondingly increase or decrease viral expression.

SRPK2 Stabilizes SR Proteins and Increases HIV Production. Several kinases, including the SRPK family kinases and Clk family kinases (Clk1–Clk4), have been reported to phosphorylate SR proteins (9, 20, 21). We recently established two SRPK2-expressing Flp-In293 cell lines (SRPK2-2 and SRPK2-3) (Fig. 6, which is published as supporting information on the PNAS web site). When these lines are transfected with HIV-1 pNL4-3, SR proteins, such as SC35 and SF2/ASF, largely avoid virus-induced degradation (Fig. 2*A*, lanes 12 and 16), and the phosphorylation status of SR proteins is maintained (detected with mAbs 104 and 1H4; Fig. 2*A*, lanes 4 and 8). Intracellular levels of hnRNPA1 and Pin1, in contrast, are not affected by SRPK2 overexpression (Fig. 2*A*, lanes 22 and 24). Transient expression of SRPK1 also increases SRp55, whereas transfection of the kinase-dead mutant (SRPK1-KM) is ineffective (data not shown), indicating that the levels of intracellular SR proteins depend on the kinase activity of SRPKs.

In addition to stabilizing SR proteins and maintaining SR phosphorylation status, overexpression of SRp75, SRp55, and SRp40 increases HIV production (measured by p24*gag* antigen levels in culture supernatants) from HIV-transfected cell lines (Fig. 2*B*). In contrast, as reported previously (22), overexpression of SF2/ASF and SC35 decreases p24*gag* levels (data not shown), suggesting that various SR proteins may play different roles in the RNA processing of HIV-1.

To evaluate the functional significance of the SRPK2–SRp75 signaling pathway for HIV propagation, SRp75 was overexpressed with NL4-3 in Flp-In293 cells or SRPK2-2 cells. Significantly, HIV-1 NL4-3 virus production is enhanced 10-fold when SRp75 is transfected into SRPK2-2 cells (Fig. 2*C*). This result strongly supports the idea that HIV-1 replication efficiency depends on SRPK-mediated stabilization of SR proteins, especially SRp75.

The Isonicotinamide Compound SRPIN340 Selectively Inhibits SRPK. The above findings suggest that a specific inhibitor of SRPK could suppress replication of HIV-1 and other viruses that require SR protein-dependent RNA processing for their propagation. This idea prompted us to use a scintillation proximity assay with a synthetic RS-repeat peptide as the substrate (20) to screen a chemical library for specific SRPK inhibitors. After screening >100,000 chemicals, we found an isonicotinamide compound, *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl] isonicotinamide (Fig. 3*A*), that potently inhibits SRPK1 kinase activity, with a K_i value of 0.89 μM (Fig. 3*B*). We named this compound SRPIN340 (for a diagram of SRPIN340 synthesis, see Fig. 7, which is published as supporting information on the PNAS web site).

SRPIN340 inhibits SRPK1 and SRPK2 but does not significantly inhibit other SRPK, such as Clk1 and Clk4 (Fig. 3*C*). In fact, when tested at concentrations up to 10 μM in an extensive examination of >140 enzymes (Table 1, which is published as supporting information on the PNAS web site), SRPIN340 failed to inhibit any additional Ser/Thr kinases. Thus, SRPIN340 is highly specific for the two kinases SRPK1 and SRPK2, which, as we show here, are central to phosphorylation and stabilization of SR proteins necessary for virus production in HIV-1 transfected or infected Flp-In293 cell lines.

SRPIN340 Inhibits SR Phosphorylation by SRPK and Promotes Degradation of SRp75. Consistent with the demonstration that SRPK-mediated phosphorylation stabilizes SR proteins (see Fig. 2*A*), SRPIN340 inhibits SR phosphorylation by SRPK in Flp-In293 cells and promotes degradation of SRp75 in a dose-dependent manner (Fig. 4). Thus, because retention of SRp75 is necessary for HIV expression, SRPIN340 emerges as a potential inhibitor of HIV production.

Initial studies using SRPIN340 to inhibit HIV replication in

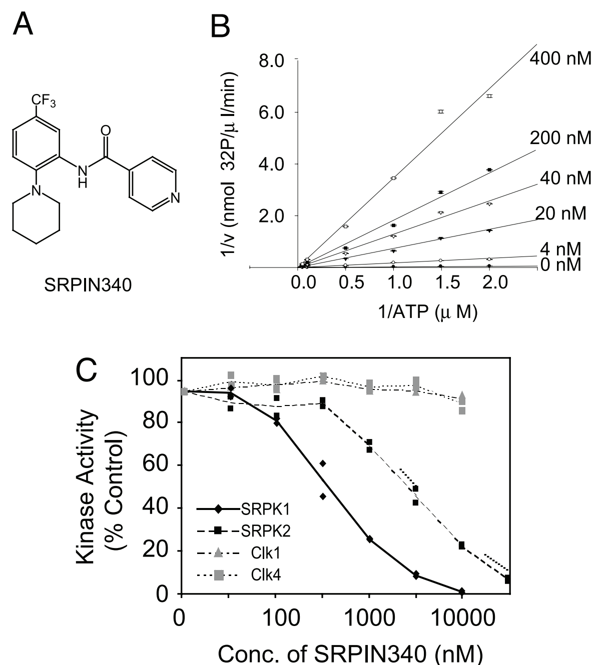

Fig. 3. Screening and characterization of SRPIN340, an SRPK-selective inhibitor. (*A*) Structures of *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide (SRPIN340). (*B*) Lineweaver–Burk plot showing the competitive inhibition of ATP by SRPIN340. SRPK1 kinase activity was measured at the indicated concentration of SRPIN340 and ATP. SRPIN340 competitively inhibits the SRPK1 kinase activity with a K_i value of 0.89 μM. (*C*) Inhibition spectrum of SRPIN340 for various protein kinases. The preparation and measurement of kinase activities of His_6-tagged mSRPK1, mSRPK2, Clk1, and Clk4 were performed as previously described (17). The average inhibitory ratios from two independent assays are shown. The inhibitory effects of 10 μM SRPIN340 on other protein kinases are summarized in the Table 1.

cell lines *in vitro* have yielded variable results. We have found that the concentration of serum used in the culture is important, as is the multiplicity of infection (data not shown). Furthermore, preliminary studies show that SRPIN340 is not an effective inhibitor of HIV replication in standard cell line infection assays commonly used as the initial screen for potential HIV therapeutics (our unpublished observation). SPRIN340 derivatives may prove more effective for this purpose and are perhaps worthwhile pursuing, because SPRIN340 itself strongly inhibits replication of another RNA virus (Sindbis).

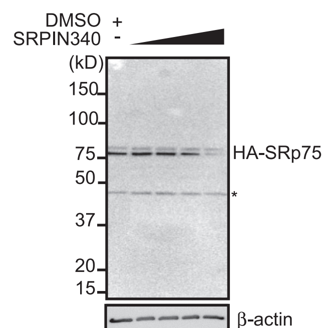

Fig. 4. Effect of an SRPK inhibitor on SR protein stability. Effect of SRPIN340 on the stability of SRp75. The HA-tagged SRp75 was expressed in HEK293 cells. At 72 h after transfection, 0, 10, 20, and 50 μM SRPIN340 was added to the culture media. After 12 h of incubation, the amounts of HA-tagged SRp75 and β-actin (control) in the whole-cell extracts were examined by immunoblotting with an anti-HA Ab or anti-β-actin Ab, respectively.

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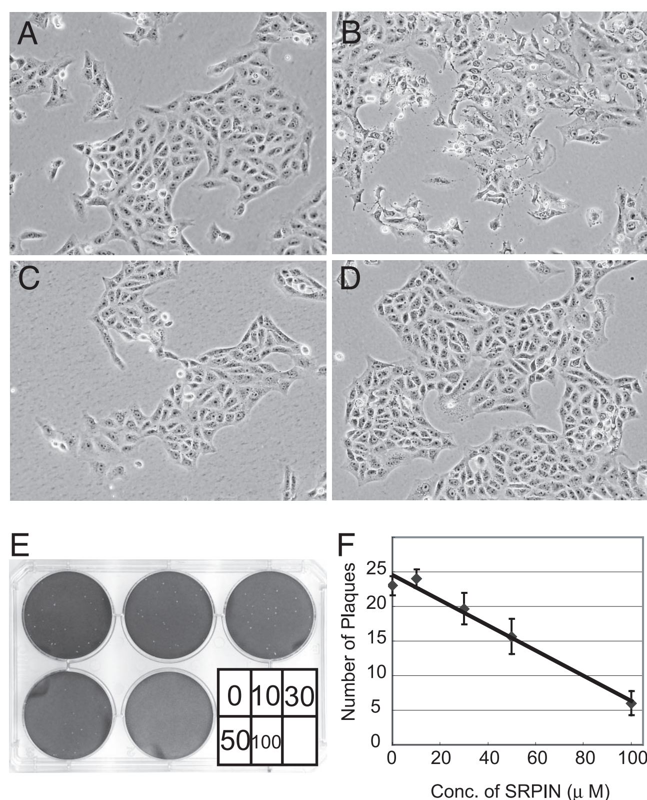

Fig. 5. SRPIN340 suppresses the propagation of Sindbis virus. The Sindbis virus (strain TE) was prepared from the culture supernatant of BHK as described in ref. 29. (*A–D*) Vero cells were mock-infected (*A* and *C*) or were infected with Sindbis virus (*B* and *D*) at a multiplicity of infection of 1–10 plaque-forming units per cell. After 20 h of incubation with DMSO (*A* and *B*) or 40 mM SRPIN340 (*C* and *D*), phase contrast images were captured. (*E*) The inhibitory effect of SRPIN340 on the Sindbis virus propagation was quantified by plaque assay. Vero cells were infected with Sindbis virus (strain TE) with the indicated concentrations (10, 30, 50, and 100 μM) of SRPIN340 or with DMSO as a control. After 4 days, titers of virus infectivity were determined as described in ref. 29. (*F*) The data of three independent assays were shown in a graph with standard deviations.

SRPIN340 Inhibits Propagation of Sindbis Virus. SRPIN340 (40 μM) rescues Vero cells from the cytopathic effect of Sindbis virus (Fig. 5*D*). In a plaque assay with Vero cells (Fig. 5*E*), the SRPIN340 IC_{50} for Sindbis virus propagation was 60 μM (Fig. 5*F*). Preliminary studies indicate that SRPIN340 also suppresses propagation of severe acute respiratory syndrome virus (data not shown). Thus, the kinase activities of SRPKs appear to be required for propagation of a variety of RNA viruses.

Because SRPIN340 suppressed proliferation of cytomegalovirus, SRPKs may also be required for propagation of DNA viruses. Consistent with this hypothesis, SR proteins purified from adenovirus- or vaccinia virus-infected cells have been shown to be hypophosphorylated and functionally inactivated as splicing regulatory proteins (23). Similarly, during herpes simplex virus 1 infection, phosphorylation of SR proteins has been shown to be reduced, with a concomitant loss of the ability of these proteins to function in spliceosomal assembly (13).

Preliminary Studies of SRPIN340 Toxicity. The rudimentary toxicity data available for SRPIN340 is promising with respect to the potential for use of this compound as a therapeutic. No adverse effects were observed when SRPIN340 was orally administrated to rats, even at the highest SRPIN340 dose (2,000 mg/kg). In this study, 10 rats were observed for 2 weeks after SRPIN340 administration. All of the rats survived, and none showed visible evidence of illness or debilitation. In addition, no abnormalities in chromosomal structure and chromosome number were observed when SRPIN340 was administrated to CHO cells in the culture medium at the highest SRPIN340 dose (5 mg/ml) for 24 h. Furthermore, no mutagenic effects were detected when SRPIN340 was exposed with *Salmonella typhimurium* at the highest SRPIN340 dose (5 mg per plate) in standard Ames tests.

Discussion

Given the necessity to encode multiple proteins in small viral genomes, it is not surprising that virus RNA expression capitalizes on the alternative splicing capabilities of the host RNA processing machinery (22, 24). Our studies confirm this viral dependency on host RNA processing and show that the kinases that activate and stabilize SR proteins, which are central to the RNA-processing machinery, offer targets for intervention in viral diseases.

Previous studies have shown that DNA virus infection (adenovirus and vaccinia virus) also induces dephosphorylation and functional inactivation of SR proteins. As a possible mechanism, Kanj *et al.* (25) reported recently that adenoviral infection causes an accumulation of ceramide, which results in the dephosphorylation of SR proteins. Because *de novo* ceramide synthesis by Fas activation or heat shock also causes dephosphorylation of SR proteins via the activation of PP1 (26), the viral infection may mimic the ceramide-medicated host pathway. In the case of adenovirus, translated viral protein E4orf4, which occurs in the early phase of the infection, binds to the host PP2A and SR proteins, resulting in the dephosphorylation of the SR protein and the subsequent IIIa splicing of the viral precursor mRNA, the dominant transcript in late phase after infection (27). These findings suggest that the dynamics of the viral life cycle may in general be governed directly by the regulation of SR protein phosphorylation in the host RNA-processing machinery.

We show here that HIV Tat, which is produced relatively early in the HIV infection cycle, may control the localization of SR proteins (Fig. 1 *Bottom*). Although the specific mechanism(s) through which this control is exerted still remains to be elucidated, our studies linking SR protein activity to effective HIV infection support the idea that SR protein activity plays a key role in controlling the viral life cycle. Previous studies have also implicated SR proteins in HIV splicing and expression by showing that overexpression of the 9G8, SC35, or ASF/SF2 SR proteins suppresses HIV production in 293T cells (23). In our Flp-In293 system, we confirm that overexpression of these SR proteins significantly suppresses HIV production (data not shown).

Overexpression of SRp40, SRp55, or SRp75 also contributes to HIV overproduction (Fig. 2*B*). The consensus binding sequence of SRp75 is not yet determined in HIV RNA. However, we have shown that activation of the SRPK2–SRp75 pathway, which may serve to constitutively activate splicing machinery, results in a striking acceleration (>20-fold) in HIV expression (Fig. 2*C*). Furthermore, the virions produced in cells overexpressing SRPK2 and SRp75 were infectious, at least as indicated by the ability to replicate and propagate in MT-4 cell lines (data not shown). Thus, we hypothesize that specific inhibition of the SRPK–SRp75 pathway may provide a strategy for controlling viral expression.

The use of SRPIN340 for this purpose may be effective for some viruses but not for others. We have shown thus far that SRPIN340 effectively inhibits Sindbis virus but that it is variably effective for HIV virus. Overall, our preliminary screens indicate that SRPIN340 is likely to be most effective for acutely replicating virus such as Sindbis, severe acute respiratory syndrome, and cytomegalovirus. Treating such acute viral infections with SRPIN340 may, in any event, be more practical. Because the

SRPIN340 targets host SR proteins, its utility as a drug will depend on finding treatment regimes that control the virus but do not significantly impact the host RNA-splicing machinery. Future studies with SRPIN340, its derivatives, and other chemicals that target SRPKs could be directed toward developing antiviral treatment regimes and drugs.

Materials and Methods

Plasmid Constructs and Cell Lines. HA-tagged SF2, SC35, SRp40, SRp55, and SRp75 were kindly provided by W. Y. Tarn (Academia Sinica, Taipei, Taiwan). Venus was kindly provided by A. Miyawaki (RIKEN, Saitama, Japan). The Flp-In System (Invitrogen) was used for the establishment of stably expressing cell lines. Flp-In293 cells contained a single, stably integrated Flp recognition target site at a transcriptionally active locus. Briefly, mouse SRPK2 (mSRPK2) cDNA (25) or Venus was inserted into pcDNA5/Flp recognition target/V5-His-TOPO (Invitrogen) and transfected into Flp-In293 cells with pOG44 (Flp recombinase expression vector), resulting in the targeting integration of the expression vector. Then Flp-In293 cells stably expressing mock (Venus) or SRPK2 (SRPK2-2 or SRPK2-3) were selected and established as instructed.

Immunostaining. Flp-In293 cells grown on coverslips in 12-well dishes were transfected with expression vectors (Quantum Biotechnologies) of GFP or GFP-Tat by using GeneJuice (Novagen) and further incubated for 72 h. Cells were fixed with 4% paraformaldehyde in 250 mM Hepes·NaOH (pH 7.4) for 20 min, permeabilized with 1% Triton X-100 in PBS for 20 min, and washed four times in PBS. The cells were incubated in blocking solution (1% BSA/0.2% gelatin/0.05% Tween 20 in PBS, pH 8.0) for 30 min and incubated with primary Ab, an anti-SC35 Ab (1:100; Sigma), in blocking solution for 2 h. After being washed four times in PBS containing 0.05% Tween 20, the coverslips were incubated with a secondary Ab, anti-mouse IgG labeled with Alexa Fluor 555 (1:2,000; Molecular Probes), in blocking solution for 2 h. After being washed several times over 1 h in PBS containing 0.05% Tween 20 and three times with PBS, the coverslips were mounted in ProLong Gold antifade reagent (Molecular Probes). The fluorescence images of Alexa Fluor 555 and GFP and the differential interference contrast (DIC) microscopy images were taken with a confocal microscope (Olympus FV1000).

Ab and Immunoblotting. Anti-phosphoSR Mab104 (1:250; American Type Culture Collection), 1H4 (1:250; Zymed), anti-SC35 (1:250; PharMingen), anti-SF2 AK96 [1:1,000; a gift from A. Krainer (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)], anti-hnRNPA1 (1:500; Santa Cruz Biotechnology), anti-Pin1 (1:1,000; Cell Signaling Technology), anti-HA (1:500; Santa Cruz Biotechnology), anti-V5 (1:1,000; Invitrogen), anti-SRPK2 (1:1,000; BD Biosciences), and anti-β-actin (1:2,000; Sigma) were used in this study. Total proteins were separated in a 4–20% gradient polyacrylamide gel (Daiichi Pure Chemicals), transferred to a nitrocellulose membrane, and probed with the Abs listed above. Signals developed by ECL (GE Healthcare) or WestDura (Pierce) were detected and captured by ChemDoc (Bio-Rad). Images were captured and quantified with Quantity ONE software (Bio-Rad).

***In Vitro* Kinase Assay and Kinetic Analysis.** His_6-tagged mSRPK1, mSRPK2, mClk1, and mClk4 were expressed in *Escherichia coli* (BL21) and purified as described in ref. 17. The enzyme and substrates were incubated with the indicated concentrations of ATP and SRPIN340 (shown in Fig. 3). SRPK1 kinase activity was measured as described in ref. 17. The inhibitory activity was analyzed by Lineweaver–Burk Plot using SigmaPlot software.

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