Inhibition of early stages of HIV-1 assembly by INI1/hSNF5 transdominant negative mutant S6

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Running title: INI1 transdominant mutant inhibits HIV-1 early assembly

Key Words: INI1/hSNF5, nuclear export, transdominant mutant, HIV-1, assembly

Word Count for Abstract:

Word Count for Text:
Abstract:

INI1/hSNF5 is an HIV-1 integrase (IN) binding protein specifically incorporated into virions. A truncated mutant of INI1 (S6, aa 183-294) harboring the minimal IN binding Rpt1 domain potently inhibits HIV-1 particle production in a transdominant manner. The inhibition requires interaction of S6 with IN within Gag-Pol. While INI1 is a nuclear protein and harbors a masked nuclear export signal (NES), the transdominant negative mutant S6 is cytoplasmic, due to the unmasking of NES. Here, we have examined the effects of subcellular localization of S6 on HIV-1 inhibition and further investigated the stages of assembly that are affected. We found that targeting an NLS-containing S6 variant [NLS-S6(Rpt1)] to the nucleoplasm (but not to nucleolus) resulted in complete reversal of inhibition of particle production. Electron microscopy indicated that while no electron dense particles at any stage of assembly were seen in cells expressing S6, virions were produced in cells expressing the rescue mutant NLS-S6(Rpt1) to wild-type levels. Immunofluorescence analysis revealed that p24 exhibited a diffuse pattern of localization within the cytoplasm in cells expressing S6 in contrast to accumulation along the membrane in controls. Pulse-chase analysis indicated that in S6-expressing cells, although Gag(Pr55\textsuperscript{Gag}) protein translation was unaffected, processing and release of p24 were defective. Together, these results indicate that expression of S6 in the cytoplasm interferes with trafficking of Gag-Pol/Gag to the membrane, and causes a defective processing leading to inhibition of assembly at an early stage prior to particle formation and budding.
Introduction:

HIV-1 integrase (IN) is a virally encoded enzyme that catalyzes the insertion of viral DNA into host chromosomal DNA (4). IN also influences stages of viral replication other than integration, as is evident from the study of IN mutants. While class I IN mutants with substitutions in the catalytic residues block proviral DNA integration, class II IN mutants are defective for viral assembly, particle production, post-entry uncoating, reverse transcription and/or nuclear import (3, 14, 21, 44, 45). The exact mechanism by which IN mutants exert these pleiotropic effects is unknown. One possibility is that these pleiotropic effects are due to defects in the interaction with IN-binding cellular proteins. IN binds directly to INI1 (Integrase interactor 1), LEDGF (Lens epithelium-derived growth factor), Transportin-SR2 and EED (embryonic ectodermal development) among others (7, 10, 19, 41). These IN-binding proteins exhibit varied functions and may account for the multifaceted role of IN in modulating HIV replication.

INI1, also known as hSNF5, BAF47 and SMARCB1, is the first IN-binding protein to be isolated and a core component of the SWI/SNF chromatin-remodeling complex (19, 42). INI1 binds tightly to IN in vitro and in vivo, is selectively incorporated into HIV-1 virions and is found in the reverse transcription and pre-integration complexes isolated from cells (17, 19, 26, 39, 46). Recombinant INI1 stimulates and inhibits in vitro integrase joining activity in a concentration-dependent manner (12, 19). Additionally, INI1 is a tumor suppressor gene that is biallelically deleted or mutated in aggressive pediatric tumors known as rhabdoid tumors (40).

INI1 contains two highly conserved domains, termed Rpt1 and Rpt2, that are imperfect direct repeats of each other and a third conserved domain termed homology...
Previously, we reported that Rpt2 harbors a nuclear export signal (NES) with a consensus motif, \( \Psi X_1 \Psi X_2 \Psi \) (where \( \Psi \) is a hydrophobic residue and \( X \) is any amino acid) that mediates nuclear export of INI1 in a CRM1-dependent manner (11). We found that hydrophobic amino acid residues within the NES sequence 266-LNIHVGNISLV-276 of INI1 are necessary for nuclear export (11). Additionally, the L266 residue of this sequence is invariant among all INI1 homologues across phyla, implying a functional significance. While the significance of NES in INI1 function is unclear, it has been demonstrated that INI1 is exported from the nucleus upon HIV-1 infection (39).

Previously, we have demonstrated that a fragment of INI1 termed S6 (aa, 183-294) harboring the minimal IN-binding Rpt1 domain acts as a dominant negative mutant and potently inhibits late events of HIV-1 replication (46). S6 also contains a part of the Rpt2 region including the NES. While full-length INI1 is a nuclear protein, S6 is cytoplasmic due to the unmasking of NES. Jurkat cells stably transfected with S6 (but not wild-type INI1 control) are protected from infection by full-length HIV-1R3B in a multicyle HIV replication (more than 15 days), demonstrating the ability of S6 to continually exert an inhibitory effect over time. Substitution mutants of S6 defective for interaction with IN are unable to mediate this potent inhibition, indicating that IN-INI1 interaction is necessary for this inhibition (47). Consistent with this idea, S6 is unable to inhibit particle production from viruses that either harbor a mutant IN defective for interaction with INI1 or contain a deletion of IN (46). Together, these results indicate that the ectopically localized fragment of INI1 harboring the minimal IN-binding domain inhibits late events, likely due to binding of IN and sequestration of Gag-Pol. However, the exact
mechanism of inhibition or the stages of late events that are blocked by S6 are not known.

Currently there are no drugs used in the clinic that specifically target the viral assembly process. The mechanistically closest therapeutic intervention is provided by protease inhibitors that affect/block maturation of assembled particles. INI1/S6 mediates potent inhibition of HIV-1 late events (100-10,000 fold) and therefore, further inquiry into the exact nature of this inhibition may lead to development of pharmacological agents that target late events of HIV-1 replication. Here, as a first step towards understanding the mechanism of inhibition of HIV-1 late events by S6, we tested to determine if cytoplasmic localization of S6 is necessary for inhibition. Recent studies from our laboratory have indicated that INI1 is a multimer and that some multimerization-defective mutants of S6 reversed the inhibitory effect (12). Because these mutants also disrupted the NES, it is unclear if the reversal of inhibition is due to a defect in multimerization and/or nuclear export (12). Furthermore, we tested to determine the stage/s of assembly that is affected by S6 using electron and fluorescence microscopy and pulse-chase analysis of Gag. Our results reveal that expression of S6 in either cytoplasm or nucleolus inhibited particle production while targeting it to the nucleoplasm abrogated the inhibitory effect. In addition, we found that S6 affects early stages of assembly such as Gag and Gag/GagPol trafficking to the membrane, and affects Gag processing. Our studies indicate that inhibition mediated by S6 affects early stages of assembly leading to inhibition of particle production.
Materials & Methods

Construction of INI1/S6 mutants. The CFP-fusions of INI1, S6 and S6(Rpt1), with and without NLS, were generated as follows: First, plasmids pCFP-NLS-INI1, pCFP-NLS-S6 and pCFP-NLS-S6(Rpt1) harboring NLS were generated by insertion of PCR-amplified INI1, S6 or S6(Rpt1) fragments, respectively, containing 5' Bam H1 and 3' Bgl II overhangs into the Bam H1 site of pECFP-Nuc (Clontech cat#8904-1). This cloning regenerated Bam H1 at the 5' end but not at the 3' end of the plasmid. To obtain CFP mutants lacking NLS, CFP-NLS clones were digested with BamH1 (at the 5' end of the insert and downstream of NLS) and Bgl II (upstream of NLS) to remove NLS and self ligated.

Transfection, viral particle production and p24 ELISA. 293T cells were transfected at 30% confluency in 6-well plates with 1.5\( \mu \)g of the transducing vector pHR’CMVGFP, the Gag-Pol expression vector pCMV∆R8.2, and the VSVg envelope expression vector pMDG, in a 2:1:1 ratio along with 5\( \mu \)g of CFP-INI1 or CFP-S6 mutants using the calcium phosphate method (Millipore cat# S-001). Media were changed 16hrs post-transfection and the supernatant and cells were collected 48hrs later. The viral supernatant was passed through a 0.45\( \mu \) cellulose acetate filter (Nalgene) and HEPES was added to neutralize the pH. Transfected cells were lysed using 80\( \mu \)l of RIPA buffer plus protease inhibitors. The intracellular and virion-associated capsid protein content was estimated by using p24 ELISA (Perkin Elmer Cat # NEK050B).

Confocal Microscopy. For analyzing the localization of CFP and GFP-fusion proteins, cells were transfected at 30% confluency in 6-well plates containing 12-mm circular
coverslips with 5 µg of DNA expressing CFP-S6 or other mutants, as described above. About 8 hr post-media change, cells were fixed with 2% paraformaldehyde for 10 min, permeabilized in 0.5% Triton x100 for 10 min and treated with 1 µg/ml RNAse A for 45 min at 37˚C. Cells were then stained with 40 µg/ml propidium iodide for 5 min and mounted on slides with mounting medium containing 1 mg/ml p-phenylenediamine and 90% glycerol. Images were captured at 63x magnification with or without a 4x zoom, using a Leica SP2 confocal microscope.

**Transmission Electron Microscopy.** 293T cells were transfected in 60mm plates with 600 ng HIV-1 R3B and 9 µg of CFP-INI1, CFP-S6 or CFP-NLS(Rpt1). Media was changed 17 hrs after addition of DNA and at 3hrs post media-change, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hour at room temperature in the dark. Cells were postfixed with 1% osmium tetroxide followed by 2% uranyl acetate, dehydrated through a graded series of ethanol and embedded in LX112 resin (LADD Research Industries, Burlington VT). Ultrathin sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate. Images were collected using a JEOL 100 CXII or JEOL 1200EX electron microscope at 80 kv.

**Immunofluorescence.** 293T cells were plated in polystyrene chamber slides (BD cat # 354114) and transfected with 1.5 µg of CFP-INI1, CFP-S6 or CFP-NLS(Rpt1) along with 100 ng HIV-1 R3B DNA. Media was changed 16 hours post-transfection and cells were incubated for an additional 24 hrs followed by fixation with Eddy fix [3.7% paraformaldehyde, 0.1% glutaraldehyde, 0.15 mg/ml saponin (Sigma cat# S-4521) in PBS] for 15 minutes at 37˚C (36). Cells were washed with Phosphate Buffered Saline (PBS) 3 times and permeabilized with 0.5% TritonX 100 for 10 minutes at 37˚C. Cells
were washed, blocked with 2.5% bovine serum (Santa Cruz Biotechnology cat # sc-2369) for 30 minutes at 37˚C and incubated with 1:500 dilution of goat α-p24 antibodies (Gift from Dr. David Ott) plus 1.5% bovine serum for 3 hours at 37˚C. After washing 3 times with PBS, samples were incubated with 3µg/ml PE(phycoerythrin)-bovine anti-goat IgG (Santa Cruz Biotechnology cat# sc-3747) plus 1.5% bovine serum for 45 minutes at 37˚C room temperature. Cells were washed 3 times and coverslips were mounted using mounting medium +/- DAPI (Vector Laboratories cat # H1200/H-1000). Cells were imaged sequentially using a Leica SP2 confocal microscope at 63x magnification with or without 4x zoom and analyzed using ImageJ software. The intracellular distribution of p24 intensity in cells expressing HIV-1R3B in the presence of CFP-INI1, CFP-S6, CFP-NLSS6 or CFP-NLSS6(Rpt1) was quantified using ImageJ (http://rsb.info.nih.gov/ij/). Additionally, the intra-cellular distribution of p24 intensity in cells expressing SG3ΔIN in the absence or presence of CFP-INI1 and CFP-S6 was quantified. An 8-pixel wide line was drawn from the plasma membrane to the interior of the cell towards the nuclear membrane. A histogram representing the intensity of p24 immunofluorescence along this line was generated for each cell. The mean intensities of fluorescence generated from at least 13 cells expressing the CFP fusions were plotted over distance in pixels from the interior of the cell to the plasma membrane. Statistical analysis was carried out using GraphPad Prism and two-way ANOVA tests (GraphPad Software, San Diego, CA).

**Pulse-Chase followed by immunoprecipitation of viral proteins.** These experiments were essentially carried out as published with minor modifications. 293T cells in 10cm plates were transfected with 15µg of three-plasmid-based HIV-1 vectors and 15µg of
pCGN-INI1 or pCGN-S6 plasmids expressing HA-INI1 or HA-S6 respectively, via the CaPO₄ precipitation method. Media was changed 16 hours after the addition of DNA and labeled with [³⁵S] Met/Cys 24 hours post media change. Transfected cells were washed in RPMI-Cys-Met (containing no cysteine or methionine, Sigma cat # R7513), incubated with the same medium at 37°C for 30 minutes to starve the cells and then the media was replaced with 2mls RPMI-Cys-Met containing 2% FBS and 250µCi/ml [³⁵S] Met/Cys (Perkin-Elmer cat # NEG072-007), and pulsed for 1 hour at 37°C. This was followed by addition of chase medium (DMEM+10%FBS+5mM Methionine+5mM Cysteine) to each plate. The culture supernatants and cell pellets were collected at 0, 1, 3 and 6 hours post chase.

To harvest the virus, culture supernatants were clarified by low speed centrifugation and passed through 0.45µm cellulose acetate syringe filter (Corning cat# 431220), followed by ultracentrifugation at 35,000rpm for 45 minutes at 4°C. The virus pellet was then subjected to a step gradient centrifugation using a 20% sucrose cushion at 35,000 rpm for 2 hours at 4°C. Viral pellets were resuspended in 1ml of lysis buffer (0.1mM EDTA, 150mM NaCl, 5mM MgCl₂, 5mM CaCl₂, 1mM DTT, 20mM HEPES-KOH pH7.9, 1% Triton X 100, protease inhibitors). The intracellular proteins were collected by washing cells once with PBS and resuspending in 1ml of lysis buffer.

Cell lysates and viral supernatants were incubated at 4°C for 1 hour with rotation and cell debris was removed by centrifugation before subjecting the supernatant to immunoprecipitation. The samples were pre-cleared using 40µl of Dynabeads Protein G (Cat# 10004D, Invitrogen) for 2 hours at 4°C with rotation. The supernatant was collected by removing the Dynabeads with magnetic separation. Immunoprecipitation
was carried out overnight at 4°C by adding 2µl of α-p24 antibodies to precleared supernatants. The next day, PBS-washed 40µl of Dynabeads Protein G was added to the samples, which was then incubated for additional 3 hours. Supernatant was removed by magnetic separation. Beads were washed five times with 500µl-1ml each of wash buffer (20mM HEPES-KOH pH7.9, 150mM NaCl, 0.1mM EDTA, 1mM DTT, 1% Triton X 100, protease inhibitors), resuspended in 20µl of PBS, followed by 20µl of 5x SDS dye, and separated on a 15% SDS-polyacrylamide gel. SDS/PAGE gels were fixed with 40% methanol, 10% acetic acid in water for 1 hr, rinsed for 10 minutes in water and treated with 25mls of 1M salicylic acid containing 2% glycerol for 2 hours. Gel was dried for 1-2 hr using a gel dryer set to 80°C, followed by autoradiography.

Results

Targeting S6 derivatives to different subcellular compartments. To determine if targeting INI1 transdominant negative mutant S6 to the nucleus alters its inhibitory effect on HIV replication, we generated a panel of S6 variants that exhibit differential sub-cellular localization. Our strategy was to delete the NES signal in the S6 fragment and/or fuse this fragment to a strong SV40-derived NLS (Nuclear Localization Signal) (Figure 1A). To facilitate visualization, the mutants were also fused to cyanine fluorescent protein (CFP) at the N-terminus (Figure 1A). We carried out immunoblot analysis to determine the stability and expression of fusion proteins in the presence or absence of a co-transfected molecular clone of HIV-1R3B (Figure 1B). Among the clones generated, six CFP-fusions including CFP fusions of INI1 (full length), S6 (the transdominant negative mutant containing Rpt1 and NES), S6(Rpt1) [containing Rpt1
but no NES], as well as the NLS-containing counterparts of these three fusion proteins (CFP-NLS fusions) were stably expressed and were utilized for further study (Figure 1B).

We first determined the localization of CFP fusion proteins in 293T cells by using confocal microscopy (Figure 1C and D). A minimum of 50-100 transfected cells each were scored by confocal microscopy and the percentages of cells exhibiting various sub-cellular distribution of INI1 and S6 mutants were determined (Table 1). We primarily observed four different types of sub-cellular localization of the various mutants, including nucleoplasmic (diffuse within the nucleus), nucleolar (strongly localized to nucleoli), cytoplasmic or diffuse (both nuclear and cytoplasmic). Full length CFP-INI1 was localized in the nucleus, as expected, and concentrated in and around areas of less condensed chromatin (Figure 1C). In contrast, CFP-S6 was localized primarily in the cytoplasm or in a diffuse pattern both in nucleus and cytoplasm (Figure 1C). Deletion of the NES in CFP-S6(Rpt1) resulted in a diffuse distribution within cells (Figure 1C). CFP-NLSINI1, while retaining its nuclear localization, co-localized with regions of condensed chromatin (Figure 1D). CFP-NLSS6 protein exhibited a strong nucleolar distribution but no cytoplasmic distribution (Figure 1D). CFP-NLSS6(Rpt1), containing an NLS and lacking an NES, localized primarily in the nucleoplasm, with subtle nucleolar distribution (Figure 1D). All NLS fusion proteins were absent from the cytoplasm, confirming the strong nature of the SV40 NLS.

**Nucleoplasmic but not nucleolar localization overcomes S6-mediated inhibition of particle production.** To assess the effect of altering S6 sub-cellular localization on HIV-1 particle production, we co-expressed INI1, S6 or its mutants in 293T cells, along
with three-plasmid-based HIV-1 vectors. A p24 ELISA was performed using cellular lysates and culture supernatants to quantify intracellular viral proteins and virion-associated proteins, respectively. Consistent with our previously published results, expression of the S6 fragment resulted in potent inhibition of particle production (~100 fold) compared to that of the control cells where full-length wild type INI1 was expressed (Figure 2A). Additionally, expression of S6 resulted in about a ~10 fold reduction in the intracellular p24 (Figure 2B). The expression of a truncated fragment of S6 devoid of NES [CFP-S6(Rpt1)] continued to inhibit viral protein synthesis and particle production, consistent with the observation that this fragment was diffuse within cells and that a substantial amount of protein was localized in the cytoplasm (Figure 2A and Figure 1C). Both S6 and S6(Rpt1) lacked an NLS and did not tightly localize to the nucleus (Figure 1C).

We then tested the effect of inserting an SV40-NLS at the N-terminus of INI1, S6 and S6(Rpt1) fragments, on HIV-1 particle production. Addition of the NLS to INI1 did not have any effect on viral protein synthesis and particle production (Figure 2A and B). NLS-S6, which was strongly localized in nucleoli, continued to inhibit both intracellular and virion-associated p24 (Figure 2A and B). Interestingly, NLS-S6(Rpt1) was localized primarily to the nucleoplasm (with no cytoplasmic distribution), did not inhibit either intracellular- or virion-associated p24 (Figure 2A and B). To establish the effect of S6 and variants on particle release, we calculated p24 release efficiency by determining virion- and cell-associated p24 and expressing virion associated p24 as percent of total p24 (cell- plus virion-associated), in control cells and in cells transfected with various fragments of S6 in each experiment. The p24 release efficiency was significantly
reduced in cells transfected with CFP-S6, CFP-S6(Rpt1) or CFP-NLSS6 as compared to that in cells transfected with control INI1 (Figure 2C). However, the p24 release efficiency was similar to or better than that of controls in cells transfected with CFP-NLSS6(Rpt1) that localized in nucleoplasm (Figure 2C). These results indicated that localization of an INI1 fragment harboring a minimal IN binding domain [S6 or S6(Rpt1)], either in cytoplasm or nucleolus, inhibited particle production and that targeting S6(Rpt1) to nucleoplasm did not inhibit intracellular viral proteins and particle release.

A C-terminal truncation of INI1 is inhibitory to particle production. To further corroborate the finding that cytoplasmic (and/or nucleolar) localization was necessary, we tested an additional set of INI1 mutants differing from each other in sub-cellular localization due to single amino acid changes in the NES (11). The fragment C19 (aa 1-276) of INI1 contains an intact N-terminal region, an IN-binding Rpt1 domain, and part of the Rpt2 domain containing the exposed NES. The C19 protein is exclusively cytoplasmic in localization due to the exposed NES. Single amino acid substitutions of the hydrophobic residues within the NES domain of C19 that aligns with the consensus sequence (L266A or I263P) result in a lack of export and localization of the mutant fragments to nucleus (11). However, substitution of the non-canonical hydrophobic residues within the NES, such as I268A, do not affect nuclear export (11). Previously, the localization studies had been carried out in HeLa cells (11). Here, we determined the localization patterns of GFP-C19 and its NES mutants in 293T (the producer cells used in this study) in the presence of HIV-1 vectors, by using immunoblot and immunofluorescence analyses (Figure 3A-D and Table 2). As expected, while GFP-C19 exhibited clear cytoplasmic localization, the mutants of C19 harboring single amino acid
changes within the NES region (L266A or I263P), localized to nucleoplasm (Figure 3D). While GFP-C19(L266A) mutant localized mostly to nucleoplasm, GFP-C19(I263P) localized to nuclear and peri-nuclear regions, but not to the cytoplasm (Figure 3D). As expected, the mutation I268A, which is not a part of the NES consensus, did not affect nuclear export properties of GFP-C19(I268A) and the mutant protein retained its cytoplasmic localization (Figure 3D).

The panel of wild type and NES mutants of C19 with differential cytoplasmic and nuclear localization was tested for their effects on viral protein synthesis and particle production. Intracellular p24 levels did not vary greatly between INI1 and the C19 mutants, suggesting that decrease in intracellular viral protein levels was a property specific to S6 (Figure 4B). Comparison of virion-associated p24 in culture supernatants indicated that cytoplasmic C19 significantly inhibited (>10 fold) viral particle production when compared with INI1 (Figure 4A). This reduction in particle production was reversed when C19 was relocalized to the nucleus with the addition of L266A or I263P substitution mutations in the NES. However, addition of the substitution mutant I268A, not in the NES consensus sequence, did not alter cytoplasmic localization of C19, and this mutant protein continued to inhibit particle production (Figure 3D and 4A). In summary, our results demonstrated that when compared to INI1, the C19 cytoplasmic mutant significantly inhibited particle production, which was reversed upon re-localization of this fragment to the nucleus (Figure 4A,C). These results confirmed our hypothesis that targeting INI1 mutants to the nucleoplasm reverses their inhibitory effects on HIV-1 particle production.
Colocalization of YFP-IN with INI1 and S6 mutants. Although all S6 mutants used in this study retained the IN-binding Rpt1 domain, they exhibited differential effects on particle release. To determine if there are differences in their localization when IN is also expressed, we co-transfected YFP-IN with CFP-S6 mutants and determined their sub-cellular localization (Table 3 and Figure 5). The percentage of cells with colocalization YFP-IN and CFP-S6 was determined by scoring individual cells, and by determining if they show overlapping localization of yellow and blue fluorescence within the same cell examined. We found that YFP-IN was nuclear when expressed either alone or with CFP (Figure 5A). When YFP-IN was co-transfected with CFP-INI1, its localization overlapped with that of INI1 within the nucleus (Figure 5A). However, when co-expressed with CFP-S6, the localization of YFP-IN was shifted to cytoplasm and it tightly overlapped with that of CFP-S6 (Figure 5A). Furthermore, YFP-IN also co-localized with all other CFP-fusion proteins of INI1 and S6 as well as CFP-NLSS6(Rpt1) mutant (Figure 5A,B). These results indicated that IN tightly co-localizes with all of the CFP-S6 mutants, irrespective of their nuclear or cytoplasmic localization (Table 3 and Figure 5).

S6 inhibits particle production by blocking early stages of assembly prior to budding. While the above series of experiments demonstrated that the cytoplasmic localization of S6 fragment was essential for its inhibitory effect, the mechanism of inhibition remained unclear. S6 may inhibit HIV-1 particle production/late events by interfering with one or more steps of assembly including translation of Gag (Pr55Gag)/Gag-Pol polyproteins, post-translational stability, processing and transport of these proteins to sites of assembly, membrane binding/accumulation, multimerization,
budding and release. To establish if there is particle formation in the cells, we first carried out ultra structural analysis of cells co-transfected with CFP-INI1, CFP-S6 or the non-inhibiting mutant CFP-NLSS6(Rpt1), along with full-length molecular clone of HIV-1 <sub>R3B</sub>, using transmission electron microscopy (Figure 6). We readily observed the presence of virions at various stages of budding and maturation in cells transfected with HIV-1 <sub>R3B</sub> alone, HIV-1 <sub>R3B</sub> with CFP-INI1, or HIV-1 <sub>R3B</sub> along with the non-inhibiting mutant CFPNLSS6(Rpt1) (Figure 6A-C). However, no detectable virions, whether immature, budding or released, were observed in cells transfected with S6 (Figure 6D). Analysis of more than a hundred cells expressing S6 indicated a lack of electron dense virus-like particles, either trapped in any compartment within the cell or released, suggesting that the defect caused by S6 is occurring at an early stage of particle production, before the visible accumulation of Gag and Gag-Pol at the plasma membrane (Figure 6D and E). Quantitation of the number of cells with electron-dense particles in each of the transfected cells indicated the absence of particles in cells transfected with S6 (Table 4).

**Expression of S6 blocks accumulation of Gag at the membrane.** To rule out the possibility that the lack of visible electron dense particle-associated structures in cells transfected with S6 was due to a lack of expression of viral proteins, including Gag and Gag-Pol in these cells, we carried out immunofluorescence analysis. Cells transfected with HIV-1 <sub>R3B</sub> and co-transfected with either CFP-INI1 (control), CFP-S6 or CFP-NLSS6(Rpt1) were stained for capsid protein using α-p24 antibodies and PE (phycoerythrin)-conjugated secondary antibodies. Cells were examined for the presence of CFP-fluorescence (indicative of INI1/S6 expression) and PE fluorescence (indicative
of Gag expression). The majority of cells transfected with HIV-1R3B alone exhibited presence of PE staining in the cytoplasm with dense staining accumulating towards the cellular periphery along plasma membrane and away from nucleus (Figure 7A). Most cells transfected with CFP-INI1 also exhibited dense PE fluorescence towards the periphery (Figure 7B and C). CFP-S6 exhibited both cytoplasmic and diffuse pattern of localization when co-expressed with HIV-1R3B (Figure 7B). Interestingly, in contrast to control cells (not expressing CFP-fusions or expressing CFP-INI1), where PE staining localized towards the periphery of the cells, PE staining in S6-transfected cells exhibited a diffuse pattern within the cytoplasm or throughout the cell (Figure 7B and C). These results indicated that although Gag/Gag-Pol was expressed in cells transfected with CFP-S6, its localization pattern was diffuse, as opposed to that in the control cells. Among the group of cells transfected with CFP-S6 and HIV-1R3B, some cells expressed Gag only (and not CFP-S6). In these cells, the p24 staining was observed at the periphery, similar to that of the HIV-1R3B control (Figure 7B). These cells served as internal controls and demonstrated that expression of CFP-S6 alters the pattern of sub-cytoplasmic accumulation of Gag. Contrary to these results, in cells transfected with CFP-NLSS6(Rpt1), Gag localization was identical to that of cells transfected with HIV-1R3B alone, where it accumulated more towards the periphery along the plasma membrane (Figure 7B and C). We also tested the effect of co-expression of NLS-S6, which localized in nucleolus and continued to inhibit particle production. We found that Gag distribution in these cells was not diffuse and its accumulation was close to the membrane (Figure 7B and C). These results indicated that a lack of Gag accumulation along the periphery was a characteristic specifically observed in cells expressing S6.
To quantify sub-cytoplasmic distribution of Gag/Gag-Pol in transfected cells, the intensity of PE fluorescence reflecting p24 staining was analyzed using Image J software. The intensity plots of PE staining, which are reflective of the strength of Gag accumulation across the cytoplasm, confirmed the visual observations. A histogram plot was generated for each cell, with mean PE-fluorescence intensity plotted over distance in pixels from the interior of the cell close to the nucleus, to the plasma membrane (Figure 7D). The mean of PE fluorescence intensities from 15-20 cells that expressed both CFP-fusion protein and p24 was determined. We found that whereas PE intensity peaked at the plasma membrane in INI1 and NLSS6(Rpt1) expressing cells, this peak was greatly reduced in S6-expressing cells (Figure 7D, \( p<0.0001 \)). The PE intensity in S6-expressing cells remained steady across the cytoplasm, suggesting a lack of accumulation of Gag at the plasma membrane (Figure 7D). These results indicated that expression of S6 altered Gag distribution within producer cells. We also tested the effect of nucleolar NLS-S6 mutant that continued to inhibit particle production on the pattern of Gag accumulation within the cytoplasm (Figure 7D). Interestingly, the pattern of Gag accumulation in NLS-S6 expressing cells was similar to that of controls, exhibiting a peak expression towards the periphery (Figure 7D). We surmised that lack of accumulation of Gag at the periphery in S6 expressing cells reflected a block in the transport of Gag and Gag-Pol to the cell membrane. Thus, our results indicated that one mechanism by which cytoplasmic S6 blocked assembly and particle production was by inhibiting trafficking of Gag/GagPol. Furthermore, our results indicated that the nucleolar NLSS6 mutant did not block transport, suggesting a different mechanism of inhibition by this mutant.
Our previous results demonstrated that the deletion of IN abrogated the inhibitory effect of S6 on particle production (46). To determine if the effect of S6 to block Gag accumulation was also depended IN, we tested the distribution of Gag when IN was deleted from Gag-Pol. Viral DNA carrying IN deletions (SG3ΔIN) was co-transfected with an empty vector or vector expressing CFP-fusions of INI1 or S6 (Figure 8). Quantitative immunofluorescence analysis indicated that Gag distribution in SG3ΔIN expressing cells in the absence of S6 was more or less diffuse in the cytoplasm with a tendency to accumulate towards the periphery (Figure 8A and B). Interestingly, whether Gag was present diffusely in the cytoplasm or showed a tendency to accumulate towards the periphery of the cells, expression of S6 had no effect on distribution of Gag in these cells (Figure 8B and C, \( p=0.4 \), not significant). These results indicated that S6 inhibited membrane accumulation of Gag and Gag-Pol via sequestering Gag-Pol by binding to IN.

**Effect of S6 on intracellular viral protein synthesis and processing.** Analysis of cell- and virion-associated p24 by ELISA indicated that S6 inhibited both intracellular viral protein levels and viral particle release (Figure 2A-C). Immunohistochemical analysis indicated that the S6-mediated block in particle release correlated with a lack of accumulation of Gag along the periphery of cells (Figure 7A-D). To determine if the reason for the observed decrease in intracellular viral protein levels was due to a defect in Gag protein synthesis, processing, and/or stability, we carried out a pulse-chase experiment. Cells were co-transfected with three-plasmid-based viral vectors along with either CFP-INI1 or CFP-S6. The cells were metabolically labeled post-transfection and the total intracellular and virion-associated proteins were collected at 0, 1, 3, and 6 hr.
after the chase and immuno-precipitated using α-p24 antibodies. The arrangement of individual proteins within Gag polyprotein is composed of MA-CA-SP1-NC-SP2-P6 proteins, which are cleaved by the virally encoded protease in an orderly and highly controlled fashion. We found that in control INI1 expressing cells, early time points indicated the presence of pr55, the processing intermediates p41 (MA-CA) and p25 (CA-SP1), and processed capsid (CA) protein, p24 (Figure 9A lanes 1 and 2). With increasing time, p41 and p25 decreased, and p24 increased in the cell lysates. Furthermore, increasing amount of p24 was found in the culture supernatants indicating assembly into virions and release (Figure 9A and B, lanes 1-4). In S6-expressing cells, both pr55 and p41 were present at levels comparable to that of the control, indicating that both Gag synthesis and at least some degree of processing were normal (Figure 9A, lanes 5-8). Intriguingly, however, in S6 expressing cells p25 and p24 proteins were severely reduced or absent, but instead, an α-p24 antibody-reactive band that is smaller than p24 was apparent (indicated by asterix in Figure 9A, lanes 5-8). Furthermore, there was a dramatic decrease in virus-associated p24 in S6-expressing cells (Figure 9B, lanes 5-8). Similar results were observed when S6 was expressed in the cells transfected with full-length molecular clone, HIV-1R3B (Supplemental Figure S3). These results suggested that there was no block to translation of Gag in S6 expressing cells, but instead there was a defect in processing and incorporation of Gag into virions, which could account for the observed decrease in cell- and virion-associated p24.
Discussion

In this study, we have found that expression of a fragment of INI1 containing the minimal IN-binding domain (Rpt1) in either cytoplasm or nucleolus inhibits HIV-1 particle production and that targeting this fragment to nucleoplasm abrogates its inhibitory effect. Further analysis indicated that there are several blocks to HIV-1 late events in cells expressing S6. EM analysis indicated that cells expressing S6 failed to show the presence of any electron dense particles either at the membrane or in sub-cellular compartments, indicating a lack of formation, mislocalization or lack of release of virions. These results suggested that the S6-mediated block is occurring before the steps of budding and release. Immunohistochemical analysis of cells transfected with S6 revealed that p24 is localized diffusely throughout the cytoplasm as opposed to a gradient localization leading to an accumulation of p24 at the cell membrane as is seen in control cells not expressing S6. These results suggested that the presence of cytoplasmic S6 directly or indirectly prevents the proper distribution of Gag and Gag-Pol within the cytoplasm. Pulse-chase experiments indicated that there was no apparent translation block in these cells, but instead there was a defect in processing and severe block in virion incorporation of Gag. Our previous studies indicated that when IN was either removed from Gag-Pol or mutated to be defective for INI1 binding (as in H12Y mutant), the S6 fragment failed to inhibit particle production (46). Thus, our current and previous results together support the hypothesis that physical interaction of S6 with IN results in destabilizing Gag and prevents its trafficking to the cell membrane, thus severely inhibiting particle production. At this point it is unclear if S6 binding directly leads to a defective processing or if it is an indirect effect of accumulation of Gag.
proteins in cytoplasm due to lack of targeting, assembly and release.

We also noted that a derivative of S6 containing both NLS and NES (NLSS6) strongly localized to nucleolus and continued to inhibit particle production. One possible explanation for this inhibition is that the nucleolar NLSS6 protein could be shuttling between nucleolus, nucleoplasm and cytoplasm and hence is able to access Gag-Pol and prevent assembly of the proteins. The shuttling could be due to the SV40 NLS fused to NLSS6, as constant shuttling between nucleolus and cytoplasm has also been observed for many nuclear and nucleolar proteins (35). The other possibility is that Gag and Gag-Pol may shuttle between the cytoplasm and nucleolus. Shuttling of Gag proteins to nucleus, although demonstrated for RSV Gag, is unclear for HIV-1 Gag (16). In the case of RSV, it has been demonstrated that nuclear transport of Gag is required for efficient viral RNA packaging. Another possibility is that inhibition of assembly by NLSS6 is not due to its ability to sequester IN but rather due to its ability to interfere with the function of another essential nucleolar-localizing viral protein such as Rev or Tat (20, 25). Further experimentation is needed to investigate the exact mechanism of inhibition mediated by the nucleolar NLSS6 fragment. Nevertheless, our studies suggest that targeting S6 to the nucleolus is yet another method to inhibit viral particle production.

Since S6 binds to IN, our results may indicate an important role for Gag-Pol in transport of viral proteins during assembly. It is possible that binding of S6 to Gag-Pol may restrict the protein from accumulating along the plasma membrane. This, in turn, may cause the Gag molecules, which bind to Gag-Pol, to also remain in cytoplasmic locations and thus preclude the transport and assembly. Indeed, perturbation of Pol
encoded proteins such as IN and RT can cause a defect in assembly and particle production. Class II mutants of IN do not affect integrase activity but affect other stages of viral replication including assembly (14, 21, 44, 45). Mutants of RT have been shown to significantly reduce virion release (8, 9). Furthermore, our results indicate that when IN is removed from Gag-Pol, there is no effect on the cytoplasmic distribution of Gag even in the presence of S6. Future experiments to directly address the role of Gag-Pol in assembly may shed light on this phenomenon.

In addition to influencing the transport of Gag/Gag-Pol, it appears that the presence of S6 affects some aspect of Gag (pr55) processing. In S6 expressing cells p25 (CA-SP1) and p24 (CA) are severely reduced. In addition, an aberrant species, smaller than p24 is present in S6 expressing cells. The exact nature of this aberrant band is unclear. However, it is possible that this smaller species results from an improper proteolytic cleavage of Gag. This smaller species neither seem to accumulate in the cells (as it’s intensity does not increase with time), nor is released into virions, suggesting that it is ultimately degraded. Therefore, it appears that expression of S6 affects Gag/Gag-Pol trafficking and some aspect of processing, which may lead to the degradation of the protein and inhibition of assembly and particle release.

How does S6, a fragment of the INI1 host protein, influence trafficking of Gag/Gag-Pol and processing of Gag? Several host factors influence late events, including protein complexes of the endosomal sorting pathway, such as TSG101, clathrin adaptor protein complexes including AP-1, AP-2, AP-3 and other proteins such as Alix and Arf (2, 5, 13, 15, 18, 23, 38). Interferon induced proteins such as Tetherin affect release of virus particles after budding (28). Furthermore, phospholipid
phosphatidylinositol-(4,5)-bisphosphate \([\text{PI}(4,5)\text{P}_2]\) also has been shown to influence assembly (29). Emerging evidence indicates that INI1 and associated SWI/SNF complex influence actin cytoskeleton organization and function. Actin and actin related proteins are physically associated with the SWI/SNF complex (22, 31, 33, 42, 43). SWI/SNF interacts with actin filaments via activation by \(\text{PI}(4,5)\text{P}_2\) (32). In addition, INI1 and SWI/SNF complexes have been demonstrated to play a role in cell migration (1, 6, 24, 34, 37). Although the mechanism by which INI1 influences actin cytoskeleton organization and cell migration is currently unknown, the above reports shed light on the possible role of INI1 in facilitating HIV-1 viral protein transport, or assembly during late events. Indeed, actin has been implicated in transport and/or assembly of viral proteins during late events and it is present within HIV-1 (27, 30). Therefore, one possibility is that INI1 may assist the Gag/Gag-Pol to transport to the membrane via the actin cytoskeleton, and the dominant negative mutant S6 may interfere with this essential function of endogenous INI1, thus indirectly inhibiting trafficking of Gag/Gag-Pol. In addition to affecting trafficking, direct binding to S6 to IN may affect the Gag-Gag-Pol organization or multimerization, and may interfere with Gag processing, leading to the lack of p25 and p24 and formation of an aberrantly processed species. Future experiments to decipher these roles of INI1 may provide new insights into the function of this protein in HIV-1 assembly and may explain the mechanism of inhibition of particle production by S6.

The discovery of S6 as a potent inhibitor of viral replication opens up an avenue to harness its therapeutic potential. The design of small peptides derived from S6 or small molecules that mimic the effect of S6 may provide invaluable tools to develop
novel therapeutic strategies that potently inhibit the early stages of assembly not only to prevent HIV-1 replication in circulating lymphocytes but also to inhibit the release of virus particles from reactivated latent reservoirs, thus keeping the virus load low enough to prevent relapse of HIV-1 infection from latently infected pools.

Acknowledgement.

This work was supported by the National Institutes of Health grant, R01 AI039951 and Irma T. Hirchl /Monique Weill-Caulier scholar award to G.V.K. J.C. is supported by the Institutional AIDS training grant, T32-AI007501. We thank Dr. V. Prasad for critically reading the manuscript, Dr. Anne Muesch for useful discussions regarding the immunofluorescence analysis, Dr. Eric Yung for generating some of the constructs used in this manuscript, X. Wu for technical assistance, and Dr. David Ott for the generous gift of α-p24 antibodies. We also thank Geoffrey Perumal and Christina Palumbo from the analytical imaging facility at AECOM, for excellent assistance with transmission electron microscopy and confocal microscopy, respectively.
References


Figure Legends:

**Figure 1: Subcellular localization of INI1 mutants.** A, Diagram illustrating CFP fusions of INI1 and truncation mutants S6 and S6(Rpt1), with and without SV40 NLS sequences. Horizontal lines in the diagram illustrate the deleted region. Rpt=Repeat; NES=Nuclear export signal; HR3=homology region 3. B, Immunoblot analysis of total proteins from cells expressing CFP-fusions of INI1 and truncations in the presence and absence of HIV-1. The blots were probed with α-GFP antibodies to detect CFP fusions. Tubulin was detected as a loading control. The asterisk above the bands indicate protein of the appropriate size. C and D, Subcellular localization of CFP-INI1 and truncation mutants in the presence or absence of NLS. C, Confocal images of cells transfected with indicated CFP-fusion constructs (C without NLS, and D with NLS). Panels indicate GFP (left column) fluorescence, Propidium Iodide (middle column) fluorescence and the overlay of the two (right column).

**Figure 2: Effect of sub-cellular localization of S6 mutant on HIV-1 viral protein synthesis and particle production.** A, Levels of virion associated p24 in culture supernatants of cells transfected with variants of S6. B, Levels of intracellular p24 in the producer cells transfected with variants of S6. C, A graphic representation of p24 release efficiency as determined by the fraction of virion- and cell- associated p24 compared to that of the total p24 based on values in A and B. The values are expressed as percentages of the total.
Figure 3: Subcellular localization of C-terminal truncation mutants of INI1. A, Diagram illustrating position of NES in INI1 and alanine and/or proline substitution of hydrophobic residues within NES. B, Schematic diagram of GFP-fusions of C19 and C19 mutants with substitutions at NES. C, Immunoblot analysis of total proteins from cells expressing GFP-fusions of C19 and C19 mutants using α-GFP antibody. α-GAPDH was used as a loading control. D, Subcellular localization of GFP-INI1/C19 and mutants with substitutions at NES. Cells transfected with indicated GFP-fusion constructs imaged by confocal microscopy. Panels indicate GFP fluorescence (left column), Propidium Iodide fluorescence (middle column) and the overlay of the two (right column).

Figure 4: Effect of sub-cellular localization of C19 mutant on HIV-1 viral protein synthesis and particle production. A., Virion associated p24 in culture supernatants of cells transfected with GFP-C19 and mutants B., Levels of intracellular p24 in the producer cells transfected with GFP-C19 and mutants. C., A graphic representation of p24 release efficiency as determined by the fraction of virion- and cell- associated p24 compared to that of the total p24 based on values in A and B. The values are expressed as percentages of the total.

Figure 5: Co-localization of INI1 and S6 mutants with HIV-1 IN. Confocal microscopic analysis of 293T cells transfected with equal amounts of YFP-IN and various CFP-fusions of INI1 or S6 mutants without (A) or with (B) NLS as indicated on
the left of the panels. Panel indicates CFP fluorescence (first column), YFP-fluorescence (second column), Propidium Iodide fluorescence (third column) and the overlay of the three (last column).

Figure 6: Ultrastructural studies to determine the effect of S6 and NLSS6(Rpt1) on HIV-1 particle production. Transmission electron micrographs of cells transfected with: (A) HIV-1\text{R3B}; (B) CFP-INI1 + HIV-1\text{R3B}; (C) CFP-NLSS6(Rpt1)+ HIV-1\text{R3B}; and (D) CFP-S6 + HIV-1\text{R3B}. Images are at 14,000 magnification.

Figure 7: Immunofluorescence analysis to determine the effects of S6 and NLSS6(Rpt1) on Gag expression and localization. A. Specificity of immunostaining by \( \alpha \)-p24 antibodies. Confocal images of cells not transfected (top row) or transfected (bottom row) with HIV-1\text{R3B} and immunostained using \( \alpha \)-p24 antibodies and PE-conjugated secondary antibodies. Left-most column, DAPI fluorescence to stain the nuclei; middle column, PE fluorescence indicating p24 staining; right column, the merge of the two. B and C. Differential sub-cytoplasmic localization of p24 in cells expressing S6 and other mutants. B., Merged confocal images (without zoom, 63x) of cells transfected with either HIV-1\text{R3B} alone or HIV-1\text{R3B} along with CFP-INI1, CFP-S6 or CFP-NLSS6(Rpt1). The CFP and PE fluorescence indicate expression of CFP-fusion proteins and p24, respectively. C. Confocal images (with 4x zoom) of cells transfected with HIV-1\text{R3B} along with CFP-INI1, CFP-S6, CFPNLSS6(Rpt1) or CFP-NLSS6, respectively and immunostained using \( \alpha \)-p24 antibodies. Left most column, CFP
fluorescence indicating expression of CFP-fusion proteins; middle column, PE fluorescence indicating expression of p24; right column, the merge of the two. D. Graphic representation of fold changes in mean p24 intensity (+/- SEM) from the nuclear membrane to the plasma membrane, with distance across the cytoplasm represented in pixels. Panels in A and C represent images at 63x magnification with 4x zoom. Panels in B represent images at 63x magnification without zoom.

Figure 8: Immunofluorescence analysis to determine the effect of S6 on Gag localization in HIV-1 lacking IN. A and B, Confocal images of cells, transfected with SG3ΔIN, SG3ΔIN+CFP-INI1 or SG3ΔIN+CFPS6, and immunostained using α-p24 antibodies and PE-conjugated secondary antibodies. A, Merged confocal images (63x) of cells. B. Confocal images (63x with 4x zoom) of cells. Left most column, CFP fluorescence indicating expression of CFP-fusion proteins; middle column, PE fluorescence indicating expression of p24; right column, the merge of the two. C, Graphic representation of fold changes in mean p24 intensity (+/- SEM) from the nuclear membrane to the plasma membrane, with distance across the cytoplasm represented in pixels.

Figure 9: Pulse chase analysis to determine the effect of S6 on Gag translation, stability and processing. A and B. SDS-PAGE analysis of the α-p24 antibody-mediated immunoprecipitations (IP) of the transfected cell lysate (A) or of the virus containing culture supernatants (B). Producer cells expressing either INI1 or S6 were
metabolically labeled with $^{35}$S, and cell- and virus- associated p24 were immunoprecipitated using α-p24 antibodies at 0, 1, 3 and 6 hours post chase. Pr55 (Gag), p41 (CA-MA), p25 (CA-p2) and p24 (CA) are indicated. Asterisks marks a species smaller than p24 that is evident in S6 transfected cells.
A. CFP-INI1  CFP-S6  CFP-S6(Rpt1)  CFP-NLSINI1  CFP-NLSS6  CFP-NLSS6(Rpt1)  Rpt1  Rpt2

B. HIV  

1 2 3 4 5 6 7 8 9 10 11 12 13

α-GFP  α-tubulin

C. CFP  CFP-INI1  CFP-S6  CFP-S6(Rpt1)  Merge

D. CFP-NLS  CFP-NLSS6  Merge

Downloaded from http://jvi.asm.org/ on November 13, 2017 by guest
A. Virion Associated p24

B. Intracellular p24

C. Virus Release Efficiency

% of total p24 recovered

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<td>S6</td>
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A. **Virion Associated p24**

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- Cytoplasmic

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B. **Intracellular p24**

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- Cytoplasmic

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C. **Virus Release Efficiency**

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A.
HIV-1R3B

B.
HIV-1R3B +
CFP-INI1

C.
HIV-1R3B +
CFP-NLS
S6(Rpt1)

D.
HIV-1R3B +
CFP-S6
A. 

B. 

IP: α-p24

S6

Hours   0     1     3     6     0    1     3     6

p24  27kD

35kD

55kD

70kD

Pr55

p41

p24

p25

**p24

p25

35kD

55kD

70kD

   1      2     3       4       5      6       7      8
Table 1. Quantitation of subcellular localization of INI1, S6 and the mutants in the presence and absence of NLS

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Table 3. Quantitation of co-localization of YFP-IN with CFP-fusions of INI1 and the mutants

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Table 4. Quantitation of Electron micrographs to determine the % of cells expressing virions

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