Characterizing the Karyophilic Property of Human Immunodeficiency Virus Type 1 (HIV-1) Integrase

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As a lentivirus, the human immunodeficiency virus type 1 (HIV-1) has the unique ability to traverse an intact nuclear envelope. This essential step is predicated upon the formation of a preintegration complex (PIC), a large nucleoprotein complex of viral and host proteins. HIV-1 integrase is a viral protein that stably associates with the PIC and has been speculated to play a key role in the PIC's nuclear entry. The karyophilic property and the nuclear import mechanism of integrase may be a vital factor in determining the import mechanism of the PIC as a whole. Our aims are to better characterize the nuclear import mechanism of integrase by determining the factors required, and to identify the region within the protein responsible for its karyophilic nature. Our study used a digitonin-permeabilized cell assay to reconstitute cytosolic factors and observe the nuclear localization of integrase. Our results showed that neither cytosolic factors nor ATP was required for integrase to be transported into the nucleus. However, nuclear import was inhibited when treated with a non-hydrolyzable GTP analog, wheat germ agglutinin (WGA), or in 4°C conditions, showing that integrase is actively imported through the nuclear pore complex. Truncated integrase variants fused with EGFP showed that the region of integrase spanning residues 149-190 was able to maintain proper nuclear import. We conclude from our results that the nuclear import of integrase is an active process that may involve a novel pathway and the karyophilic determinant of integrase maps to the 149-190 region of the core domain.

Keywords: *HIV-1, integrase, nuclear import, karyophilic property, digitonin*

INTRODUCTION

By the end of 2003, a reported 20 million people have died from the acquired immune deficiency syndrome (AIDS). In 2005, 39.4 million people worldwide were living with the virus that leads to AIDS, the human immunodeficiency virus (HIV) (20). To this date, scientists are still trying to understand and characterize the mechanisms of this virus. There are two known strains of HIV in the human population: HIV-1 and HIV-2, which differ by one viral protein (15). For years, there have been numerous endeavors by scientists to develop a better understanding of the virus at the molecular level with the hopes of finding ways to suppress this killer.

HIV is a retrovirus, meaning that it stores its genomic information in the form of RNA, which then gets reverse transcribed to a duplex DNA (10). HIV more specifically belongs to the lentivirus subfamily, which means "slow" viruses. One unique feature of lentiviruses is the ability to infect non-dividing cells such as macrophages and resting T lymphocytes (6, 17). Whereas oncoretroviruses require the disintegration of the nuclear envelope during mitosis in order to gain access to the host DNA, lentiviruses are able to traverse an intact nuclear envelope of the infected host. This entry into the nucleus is a pivotal step for carrying out a productive infection in non-dividing cells. The nuclear import of HIV is one of the most controversial topics in this field, as nothing concrete has yet been confirmed about the participants, the interactions, or the occurrences that take place during this event (1, 2, 4).

Transport across the nuclear envelope is a very selective process. Ions and small molecules of up to about 40 kilodaltons (KDa) are able to passively diffuse into the nucleus (16). However, larger molecules are transported through nuclear pore complexes (NPCs) that stud the nuclear envelope. NPCs have an approximately 25-nanometer diameter diameter channel and consist of 50 to 100 different proteins collectively called nucleoporins (8). NPCs serve as selective mediators, facilitating both the

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import and export of proteins. Proteins that are given access through NPCs usually have a canonical nuclear localization signal (NLS), which generally consists of a string of basic amino acids.

Integrase is an essential, stable component of the PIC and is one of the candidates under investigation for guiding viral nuclear import. However, this role continues to remain under great controversy. The viral protein consists of three domains: the N-terminus, the core, and the C-terminus. The N-terminus domain (residues 1-51) consists of a zinc-binding motif that promotes multimerization and enhances *in vitro* activities; the core domain (residues 52-210) consists of a catalytic motif to conduct the protein's catalytic activity, and the C-terminus domain (residues 220-288) is responsible for non-specific binding to the viral DNA (9). Integrase itself is known to be karyophilic, but the region of the protein responsible for this has not yet been identified (6, 13).

This karyophilic nature of integrase suggests that it could possibly contribute to the karyophilic nature of the pre-integration complex (PIC), a large complex that is composed of viral proteins and viral DNA (3, 11). It is approximately 56 nanometers in diameter, which is over twice as large as NPCs (14). The question then arises as to which viral or cytosolic components, if any, direct or assist in this import step.

Our aim is to study and better understand two aspects about the nuclear import process of HIV-1 integrase. The first area of study is to identify the minimal region needed for nuclear import. DNA plasmids with truncated variants of integrase, fused with enhanced green fluorescent protein (EGFP) and glutathionine-S-transferase (GST), were generated. These plasmids were transfected into Hela cells and the subcellular localization of the fusion protein was observed by immunofluorescence. Results show that the region spanning amino acid residues 149-190 of HIV-1 integrase continued to localize to the nucleus, whereas residues 51-148 and 191-234 exhibited a more diffuse expression throughout the cell. The karyophilic determinant of integrase is believed to be contained within the core domain of the protein, and possibly within the 149-190 region, but has yet to be fully characterized.

The second aim of this project is to characterize integrase's mechanism of nuclear import. A digitonin semi-permeabilized cell assay was used to identify the minimal contents needed to reconstitute nuclear import. This is an *in vitro* system that has been well established for studying nuclear import factors and pathways (18). It involves depleting the cytosolic contents of mammalian cells and then reconstituting select nuclear import factors and energy to identify which factors are needed for nuclear import (Figure 2). Results show that unlike other nuclear proteins, integrase is able to be transported into the nucleus in the absence of cytosolic factors or a renewable energy source. This differs from the classical method of nuclear import, which requires the presence of soluble cytosolic import factors to bind to the import substrate. Integrase also seems to be translocated into the nucleus through an active transport mechanism, as the protein's import was inhibited by wheat germ agglutinin (WGA), at 4ºC, and a non-hydrolyzable GTP analog.

This knowledge about integrase may lead to a deeper insight about nuclear import of the PIC. The nuclear import of viral DNA and proteins is an important step in the infection of non-dividing host cells such as macrophages. Without this step, a productive infection cannot proceed. Characterizing one protein that may be involved can lead to a better understanding the characteristics of this complex entity and future steps can be taken to halt progression of the virus.

MATERIALS AND METHODS

Cloning of EGFP-GST-integrase fusion proteins. A SV40 large T-antigen NLS with EGFP and GST (Figure 1A) and a GST-EGFP fusion construct (Figure 1B), to serve as the positive and negative controls, respectively. Full length HIV-1 integrase fused with EGFP in the pCMX vector was graciously given to us from Takao Masuda. GST was then added in between the EGFP and HIV-1 integrase, flanked by SalI sites to increase the size of the protein and ensure that passive diffusion of the fusion protein does not occur. This was done with a 5'SalIGST primer and 3'SalIGST through PCR (Table I). The GST sequence had 21 nucleotide flexible linker regions on each end, was cloned into a TOPO TA vector (Invitrogen Corp., Carlsbad, CA), excised and purified with a gel extraction kit (Qiagen, Chatsworth, CA), and inserted into the plasmid to make a EGFP-GST-HIV integrase fusion construct (Figure 1C).

Cloning of truncated integrase fusion proteins. To generate truncated residues 51-148 of HIV integrase, a 5'SalILnkrHIN51 primer and a 3'BamHIHIN148 primer were used to create the 51-148 fragment

Table I. DNA Sequences of PCR primers used to generate fusion proteins consisting of wild-type or truncated HIV-1 integrase

Primer name	Primer Sequence
5'SalIGST	5'-TCGGTCGACGGGCGCGGGTGCTGGAGCAGGAGCAATG-3'
3'SalIGST	5'-TCGGTCGACCCTTTTGGAGGATGGTCGCCACCA-3'
5'SalILnkrHIN51	5'-GTCCAGTCGACGTCGGCCATAGCGGCCCATGGACAAGTA-3'
3'BamHIHIN148	5'-ATAGGATCCTTATTGACTTTGGGGATTGTAGG-3'
5'SalILnkrHIN149	5'-GTCCAGTCGACGTCGGCCATAGCGGCCGGAGTAATAGAATCT-3'
3'BamHIHIN190	5'-ATAGGATCCTTACCCCCCTTTTCTTTT-3'
5'SalILnkrHIN191	5'-GTCCAGTCGACGTCGGCCATAGCGGCCATTGGGGGGTACAG-3'
3'BamHIHIN234	5'-ATAGGATCCAACTGGATCTCTGCTGT-3'

flanked by SalI and BamHI sites. This region was amplified through PCR, and was reinserted into a pCMX vector with EGFP and GST (Figure 1D). The 149-190 region was created with a 5'SalILnkrHIN149 and 3'BamHIHIN190 primer in a similar manner (Figure 1E), as the 191-234 region was created with a 5'SalILnkrHIN191 and 3'BamHIHIN234 primer (Figure 1F); both were cloned into pCMX in the same manner (Table I). All primers were generated by Operon (Alameda, CA).

Cell Transfections Hela cells were grown on glass coverslips (22mm^2; Corning, Big Flats, NY) in 6 well plates in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (HyClone, Logan, UT), 100 units of penicillin/ml, and 0.1mg of streptomycin/ml (Mediatech, Inc.). Upon 50% confluency, plasmids were transfected with Polyfect (Qiagen) as outlined in the manufacturer's protocol. Upon a twenty-four hour transfection, the medium was aspirated and the cells were washed once with phosphate-buffered saline (PBS) and treated with a fixative solution (2% formaldehyde and 0.05% glutaraldehyde in PBS). The coverslips were then treated with mounting solution containing 4'-6-Diamidino-2-phenylindole (DAPI) (Vectashield, Burlingame, CA) and mounted onto slides. The edges were sealed with nail polish. The subcellular localization of the fusion protein was visualized through fluorescent microscopy with the application PictureFrame.

Digitonin Semi-permeabilized Cell Assay. This is a well-established *in vitro* assay convenient for studying the pathways of cellular nuclear transport. Hela cells were grown on glass coverslips (22mm^2; Corning) in 6 well plates in DMEM (Mediatech, Inc.). Upon 70% confluency, the medium was aspirated and the cells were washed with 1mL of buffer A solution (20mM HEPES-KOH pH 7.3, 110mM potassium acetate pH 7.5, 1mM EGTA pH 7.0, 2mM magnesium sulfate, 2mM DTT, 100mM PMSF, 0.5mg/ml pepstatin, 1mg/ml aprotinin, 1mg/ml leupeptin) on ice. The cells were then exposed to 35 µg/ml of digitonin detergent in buffer A solution for 5 minutes at 4˚C. Under these conditions, digitonin permeabilizes the plasma membrane, but leaves the nuclear envelope intact. (Reactions that required pre-treatment, such as with

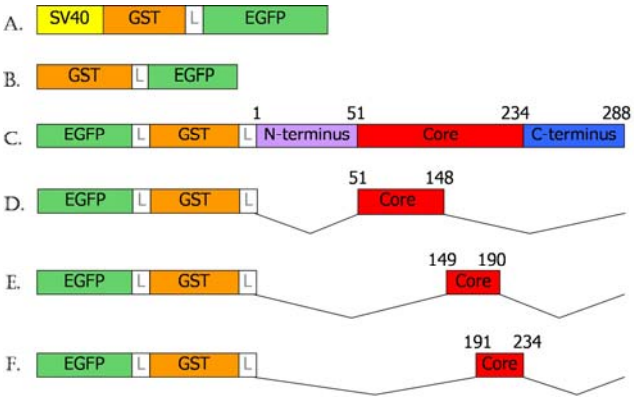

Figure 1. Constructs of Truncated Integrase Fusion Proteins (A) The NLS of SV40 was fused to GST and EGFP to serve as the positive control and (B) a GST-EGFP fusion construct was also created as a negative control. (C) Full length HIV-1 integrase fused to EGFP and GST (D) Truncated variants of HIV-1 integrase were also made: residues 51-148 of integrase fused to EGFP and GST (E) residues 149-190 of integrase fused to EGFP and GST (F) and residues 191-234 of integrase fused to EGFP and GST.

WGA, were carried out for 15 minutes at 30˚C after exposure to digitonin.) The cells were then washed with 1mL of buffer A solution. A variety of conditions were employed to manipulate the nuclear import system. This included reconstituting the Hela cytosolic extracts, a renewable energy source (0.4mM ATP, 2mM creatine phosphate, 8units/ml creatine phosphokinase), certain select import factors, energy analogs, and the protein of interest (Figure 2). The reaction was allowed to proceed for 30 minutes at 30˚C and was stopped with the addition of buffer A solution. The cells were then washed twice with 1mL of PBS and subsequently treated with a fixative solution for 15 minutes, washed again with PBS, treated with mounting solution (Vectashield), and mounted onto slides.

The control used for this assay was the bovine albumin serum (BSA) protein conjugated to an SV40 large T-antigen NLS peptide (TPPKKKRKV) and fluorescent Tetramethyl Rhodamine Iso-Thiocyanate (TRITC) molecules. 2.5µg/ml of this protein was used per reaction. 2.5µg/ml of purified recombinant integrase was used for each reaction.

Reactions with HIV-1 integrase were subjected to indirect immunofluorescence. These slides were treated with 1% BSA for 30 minutes, followed by a 1 hour incubation with primary anti-integrase residues 142-153 antibody that was diluted 1:50, and then a 1 hour incubation with a secondary goat anti rabbit that had been diluted 1:200. Mounting solution containing DAPI (Vectashield) was then added to the coverslips, which were then mounted onto slides. The edges were sealed with nail polish. The subcellular localization of the fusion protein was visualized through fluorescent microscopy with the application PictureFrame

Preparation of Hela cytosolic extracts.

Hela cells were grown in twelve 175cm^2 flasks in DMEM for approximately 5-6 days. Upon approximately 80% confluency, the media was removed and the cells were washed twice with PBS. The cells were trypsinized, washed once again with PBS, spun down, resuspended in lysis buffer (5mM HEPES pH 7.5, 6mM potassium acetate pH 7.5, 2mM magnesium acetate, 1mM EGTA, and 2mM DTT), and incubated for 10 minutes on ice. The suspension was then homogenized to disrupt the plasma membrane. The homogenate was resuspended with 10x transport buffer (20mM HEPES pH 7.5, 110mM potassium acetate, 2mM magnesium acetate, 0.5 EGTA, and 1mM DTT, 1µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml PMSF). The lysate was spun at 40,000g for 30 minutes to remove nuclei. The supernatant was then spun in an ultracentrifuge at 100,000g for 1 hour, and the resulting supernatant was then aliquoted and stored at -80ºC.

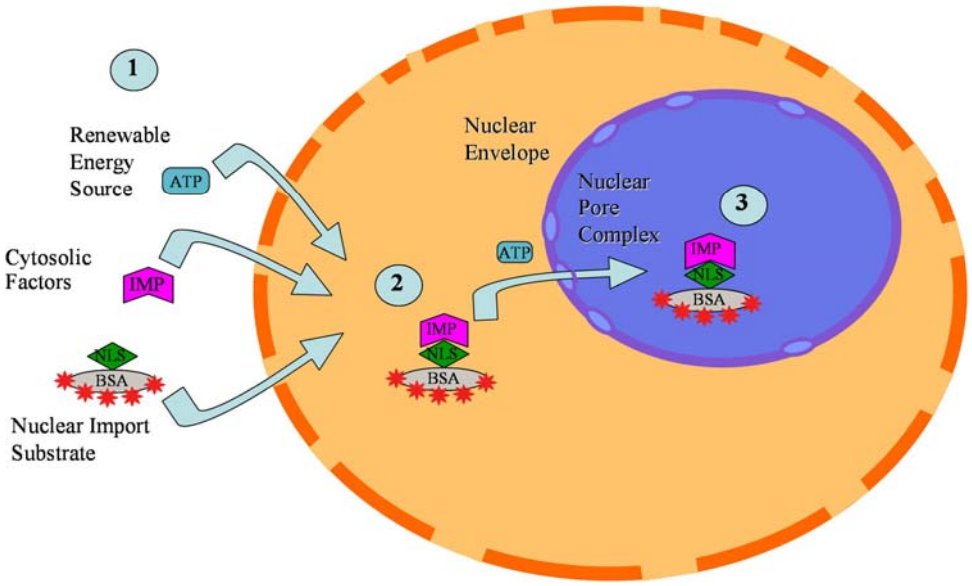

Figure 2. A schematic representation of the Digitonin Semi-permeabilized Cell Assay. Upon treatment with digitonin detergent, the cellular envelope is permeabilized. (1) Various contents, including the nuclear import substrate are then added, and (2) the reaction is allowed to proceed within the cell for 30 minutes at 30ºC, whereupon the import substrate may or may not be imported into the nucleus pending on the conditions.

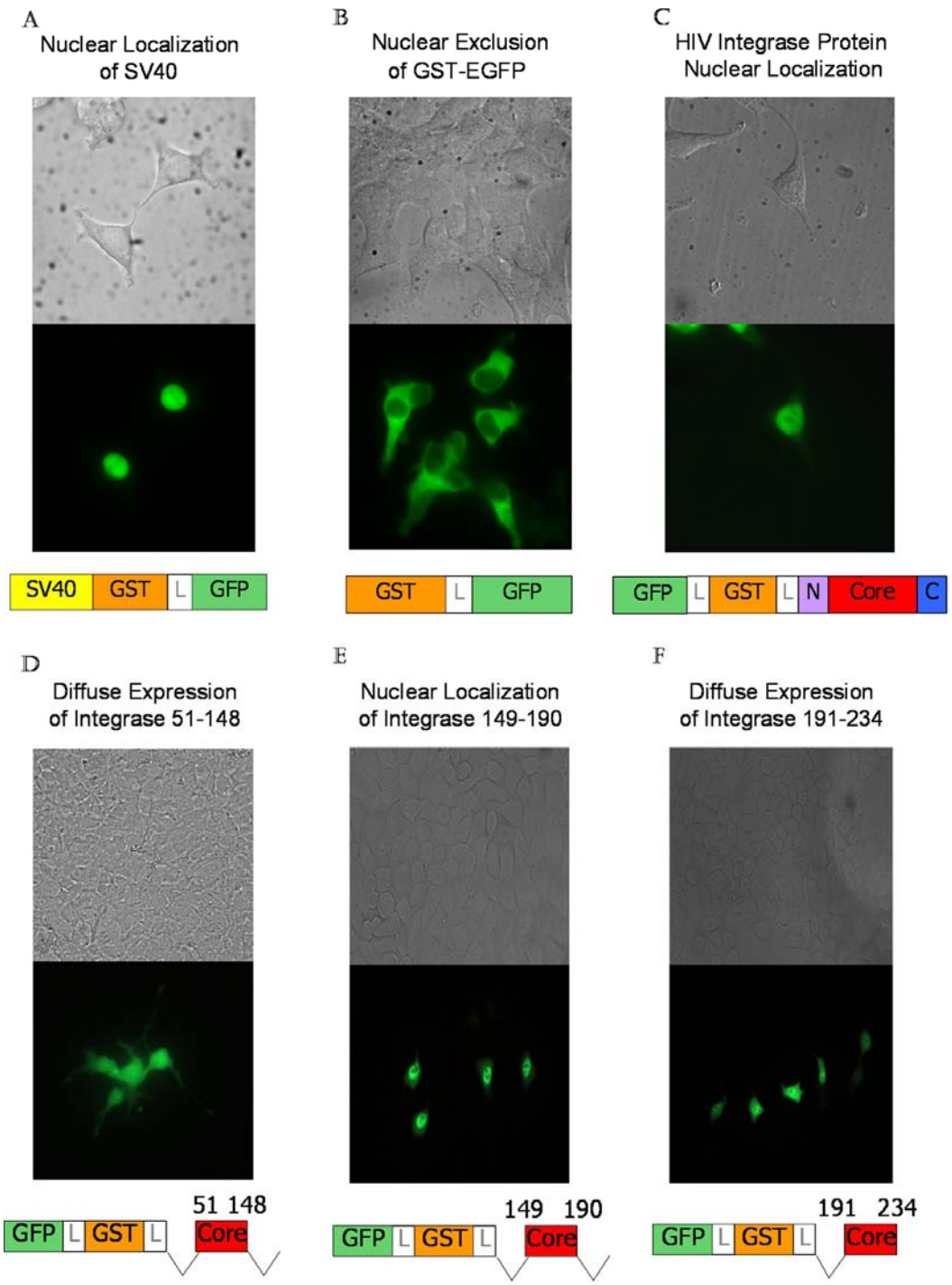

Figure 3. Transfection assays show that HIV-1 integrase is a karyophilic protein Various plasmid DNA constructs with integrase fused with EGFP and GST was generated and transfected into Hela cells. (A) Fluorescent microscopy of the NLS portion from SV40 fused to EGFP and GST localized to the nucleus, (B) while a negative control of EGFP fused to GST is excluded from the nucleus. (C) A fusion construct of full length HIV-1 integrase fused to EGFP shows nuclear localization of the fusion protein. (D) The truncated HIV-1 integrase fusion protein showed diffuse expression of the protein, (E) while HIV-1 integrase residues 149-190 fused to EGFP and GST continues to show nuclear localization. (F) The fusion protein of residues 191-234 expresses in a more diffuse manner throughout the entire cell. A differential interference contrast (DIC) image accompanies each fluorescent image in order to show the morphology of the cells.

RESULTS

HIV-1 integrase is karyophilic. The SV40 large T-antigen NLS is known to exhibit a strong karyophilic property, and the fusion protein should thus localize to the nucleus, as it does (Figure 3A). This was the positive control, and its proper nuclear localization indicates that our transfection assay is an effective way to visualize nuclear proteins. A GST-EGFP fusion protein alone should not exhibit any karyophilic nature; as expected, this fusion protein remained in the cytoplasm and shows clear nuclear exclusion (Figure 3B). This demonstrates that neither GST nor EGFP contributes any karyophilic nature to our fusion proteins. Our preliminary transfection results show that integrase is in fact karyophilic. The EGFP-GST-integrase fusion protein localized to the nucleus (Figure 3C), similar to the positive control.

HIV-1 integrase's karyophilic property lies within residues 149-190. The core region of HIV-1 integrase has been the region that has been implicated to be important for nuclear import. Thus the core was chosen to be segmented to study each region independently. The segments were chosen carefully, as to not disrupt any secondary alpha helices and beta strands, and minimally disrupt tertiary structures. The core region (residues 51-234) was truncated into the regions 51-148, 149-190, and 191-234. An EGFP-GST-integrase fusion protein of residues 51-148 show diffuse expression of the fusion protein (Figure 3D), while residues 149-190 of HIV-1 integrase continue to exhibit nuclear localization (Figure 3E). Lastly, residues 191-234 of HIV-1 integrase show a more diffuse protein expression (Figure 3F).

Integrase does not require cytosolic factors or additional energy for nuclear import. *In vitro* digitonin semi-permeabilized cell assays show that HIV-1 integrase is able to localize within the nucleus without the need for cytosolic factors or an additional renewable energy source. The NLS sequence (TPPKKKRKV) conjugated on the control protein is from an SV-40 T-antigen that exhibits strong karyophilic properties. Under full restoration of cytosolic contents and additional renewable energy source, the NLS-BSA-TRITC protein localizes to the nucleus, as expected (Figure 4A). Under the same conditions, HIV-1 integrase was observed to localize to the nucleus as well (Figure 4B).

However, in the absence of cytosol, when the reaction was given just a renewable energy source, NLS-BSA-TRITC was not retained in the cells and remained diffuse throughout (Figure 4C). This is appropriate because there are no cytoplasmic contents to retain the protein within the cells. HIV-1 integrase,

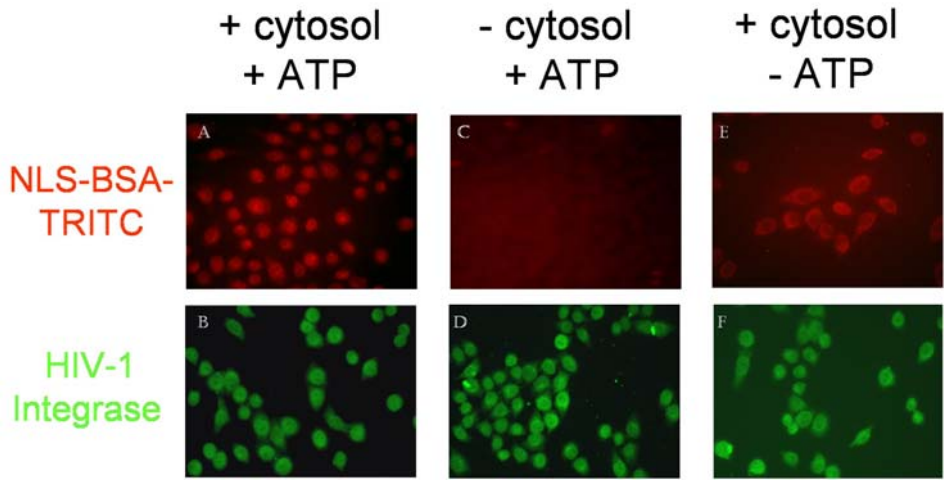

Figure 4. Digitonin-permeabilized cell assay shows that HIV-1 integrase is able to enter the nucleus without the replenishment of soluble cytosolic factors or a renewable energy source (A) The nuclear import of the NLS-BSA-TRITC conjugated protein and (B) the nuclear import of HIV-1 integrase, upon reconstitution of Hela cytosolic extract and additional energy. (C) The NLS-BSA-TRITC was not retained within the cells in the absence of cytosol, while (D) HIV-1 integrase was still able to localize within the nucleus. In the case where cytosolic factors were reconstituted, but not additional energy, (E) the NLS-BSA-TRITC protein was excluded from the nucleus, but (F) HIV-1 integrase continued to exhibit its same karyophilic property.

however, continued to exhibit a karyophilic property and localized within the nucleus (Figure 4D).

When cytosolic contents were reconstituted, but lacked additional energy, the NLS-BSA-TRITC protein remained within the cytoplasm, but was not able to enter the nucleus (Figure 4E). It requires a source of energy for nuclear import to occur. Nevertheless, HIV-1 integrase, even without additional energy, continued to display the same localization pattern as before (Figure 4F). This leads to the idea that HIV-1 integrase may not require soluble cytosolic factors such as importin α and importin β, which are the key factors for conducting nuclear import of most karyophilic proteins, in its import mechanism. Indeed, HIV-1 integrase may be using a novel pathway that is independent of these cytosolic factors to gain entry through the nuclear envelope.

HIV-1 integrase nuclear import is an active process . To investigate HIV-1 integrase's mode of transport, reactions were pre-treated with WGA, a 36 kilodalton carbohydrate binding protein that binds to sugar residues predominately found on membranes. WGA binds to NPCs, essentially "clogging up" these complexes and inhibiting macromolecules from actively entering or exiting the nucleus through these pores. The NLS-BSA-TRITC fusion protein is a relatively large protein that depends on NPCs for active transport into the nucleus. As expected, WGA inhibited the nuclear localization of NLS-BSA-TRITC, as shown by the perinuclear accumulation of the protein (Figure 5A). A majority of the protein appear to be localized along the nuclear envelope, unable to enter the nucleus. HIV-1 integrase similarly localized in the same manner (Figure 5B).

Low temperature can also inhibit active transport. A set of digitonin semi-permeabilized cell assay reactions was conducted at 4˚C, and again, the NLS-BSA-TRITC protein localized along the periphery of the nuclear envelope (Figure 5C), as was the same case for HIV-1 integrase (Figure 5D). Together, these experiments suggest that, like NLS-BSA-TRITC, HIV-1 integrase relies on NPCs to enter the nucleus.

The nuclear import process requires source of energy for the importin family members to recognize, bind to, and guide import substrate proteins through NPCs. With the addition of a non-hydrolyzable GTP analog, the NLS-BSA-TRITC was inhibited from entering the nucleus, resulting in a perinuclear localization around the nuclear envelope (Figure 6A). HIV-1 integrase similarly was unable to be translocated as well (Figure 6B).

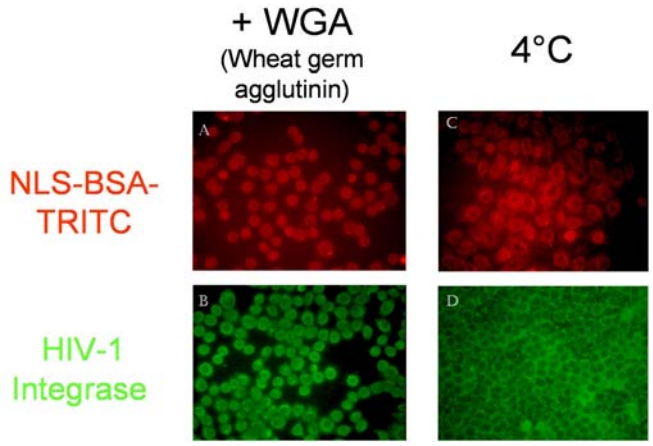

Figure 5. Nuclear import of HIV-1 integrase is inhibited by WGA and low temperature. (A) Pretreatment with wheat germ-agglutinin (WGA) inhibits the active transport of NLS-BSA-TRITC into the nucleus, as well as (B) the nuclear transport of HIV-1 integrase. (C) When the digitonin assay was performed at 4ºC, both the NLS-BSA-TRITC protein and (D) HIV-1 integrase were excluded from the nucleus in a similar fashion.

DISCUSSION

HIV-1 integrase is an accessory protein important for the integration of viral DNA into host chromosome. Additionally, due to its karyophilic nature (6, 13), it has also been strongly implicated to be a major contributor in the nuclear import of the viral PIC. We have demonstrated that HIV-1 integrase is a karyophilic protein that subcellularly localizes to the nucleus in both the transfection and digitonin semi-permeabilized cell assays, due to its karyophilic property that possibly lies in the 149-190 region of the core domain.

HIV-1 integrase's NLS is implicated to be located within the core domain, but whether this is a canonical sequence or structural feature remains unknown. As in the case with feline immunodeficiency virus (FIV) integrase, which does not appear to posses any canonical NLS, but instead contains a highly conserved zinc-binding motif in the N-terminal that is necessary for multimerization of the protein and functioning of its karyophilic property (19). However, this does not seem to be the case with HIV-1 integrase. HIV-1 integrase also has a zinc-binding

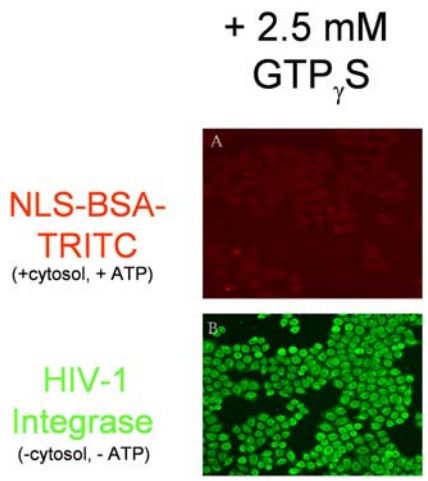

Figure 6. The addition of a non-hydrolyzable GTP analog continues to block nuclear import of HIV-1 integrase. (A) Digitonin semi-permeabilized cell assay conducted with the addition of a non-hydrolyzable GTP analog inhibited nuclear import of NLS-BSA-TRITC and (B) HIV-1 integrase.

motif, but this motif did not show to contribute to the karyophilic property of HIV integrase as in FIV integrase (19).

Residues 51-234 span the core domain, which is where the karyophilic property is deemed to be located. More specifically, residues 149-234, which constitutes the later part of the core domain, has been implicated by some groups to harbor a possible karyophilic determinant (1). Our results show that it is in fact this smaller 149-190 truncated region of HIV-1 integrase that is able to sustain nuclear import, while both the fusion proteins with 51-148 and 191-234 truncated regions of HIV-1 integrase were diffuse throughout the cell. This dispersed nature may have been contributed by degradation of the fusion protein. Western blots showed the fusion proteins had both full length and degraded proteins (data not shown). This suggests that the karyophilic property of integrase may be harbored within residues 149-190. Further truncations of residues 149-190 will be conducted to further characterize this region.

The second aim of this project was to determine the cytosolic factors, if any, needed to support nuclear import of HIV-1 integrase. A digitonin semi-permeabilized cell assay was chosen because it is a well-established method for determining such factors. The conjugated positive control, the NLS-BSA-TRITC protein, should follow the classical nuclear import pathway. It also ensures that the conditions chosen were sufficient to support nuclear import, if it were to occur. As was shown, the NLS-BSA-TRITC protein requires soluble cytosolic factors and energy to localize to the nucleus. In the classic model of nuclear import, importin factors interact with the import substrate protein, which is then directed to NPCs by Ran (8). This entire system requires energy, provided by ATP and GTP, to continually replenish the system. Our results showed that without these factors, NLS-BSA-TRITC was not able to be imported into the nucleus. HIV-1 integrase, on the other hand, was able to be imported into the nucleus both in the presence and absence of these previously mentioned factors. HIV-1 integrase does not seem to follow to the classical way proteins enter the nucleus; it is able to bypass the need for cytosolic factors and ATP replenishment.

Further characterizing this pathway led to find out what could inhibit nuclear import of HIV-1 integrase. Digitonin assays were performed with reagents and conditions that inhibit active transport through NPCs. One such reagent was WGA, which blocks active transport by physically obstructing the pores. Physical obstruction would definitely inhibit large proteins, such as the NLS-BSA-TRITC fusion protein and HIV-1 integrase from entering the nucleus. Such a phenomenon was observed when cells were pretreated with WGA; fluorescent localization of NLS-BSA-TRITC showed that the protein remained in the cytoplasm or on the nuclear envelope, suggesting that nuclear import of the conjugated protein was hindered. Likewise, HIV-1 integrase's nuclear import was inhibited with the addition of WGA, implying that it too needs NPCs to enter the nucleus. This was corroborated by subjecting the assays to low temperature, which impedes active transport through NPCs. Both proteins localized in a perinuclear fashion, unable to enter the nucleus.

In order for the nuclear import cycle to continue, Ran must be recycled back to the cytoplasm. This is achieved through a GTP-driven process, where the hydrolysis of GTP into GDP makes Ran available for binding with importin β in the cytoplasm. GTP is certainly needed in the classical import of NLS-BSA-TRITC, but we also have results that show that it is needed for HIV-1 integrase nuclear import as well. With the addition of a non-hydrolyzable GTP analog, nuclear import of both NLS-BSA-TRITC and HIV-1 integrase were defective.

Results from these various digitonin assays show that HIV-1 integrase behaves differently from the

typical nuclear import pathway of other nuclear proteins. The protein may be involved in a novel pathway that bypasses these cytosolic factors, but still relies on NPCs for passage. For instance, HIV-1 integrase may directly interact with the NPCs and gain entry directly, but that is an aspect that has to be further investigated.

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