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A peptide derived from the N-terminal region of HIV-1 Vpr promotes nuclear import in permeabilized cells: elucidation of the NLS region of the Vpr

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Abstract Viral protein r (Vpr), a HIV-1 auxiliary protein which mediates nuclear import of the viral preintegration complex (PIC), contains two regions, N- and C-terminal, which have been proposed to function as a nuclear localization signal (NLS). We have synthesized peptides corresponding to both regions (designated as VprN and VprC), conjugated them to bovine serum albumin (BSA), and tested their ability to mediate nuclear import in permeabilized cells. Only VprN, and not VprC, functioned as an active NLS and promoted translocation of the conjugate into nuclei. Nuclear import of the conjugate was found to be energy and temperature dependent and was inhibited by wheat germ agglutinin (WGA). However, it did not require the addition of cytosolic factors and was not inhibited by the prototypic SV40 large T-antigen NLS peptide. Our results show that Vpr harbours a non-conventional negatively charged NLS and therefore suggest that Vpr may use a distinct nuclear import pathway.

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Key words: Nuclear localization signal; Human immunodeficiency virus type 1; Viral protein r; Synthetic peptide

1. Introduction

During the last few years it became clear that human immunodeficiency virus type 1 (HIV-1) differs from other retroviruses in its ability to infect terminally differentiated macrophages, namely non-dividing cells [1,2]. This function has been attributed to the action of several karyophilic proteins which are present in its preintegration complex (PIC). At least three HIV-1 proteins are involved in nuclear import of the PIC, thus displaying partially redundant nuclear localization activity: the HIV-1 matrix protein (MA), a nucleocapsid component [3], the auxiliary protein viral protein r (Vpr) [4,5], and the viral integrase [6]. The requirement for MA can be observed only in viruses lacking Vpr and the converse is also true; the Vpr nuclear import function is revealed only in the background of MA mutants lacking the nuclear localization signal (NLS) function [4,5,7]. In spite of these reports, the role of MA in promoting nuclear import of the viral PIC is controversial. Recently it has been demonstrated that its NLS is 'weak' [7–10] and it requires the co-participation of Vpr in mediating nuclear import of the PIC. The viral endonuclease, integrase, which is required for chromosomal insertion of the viral genome, is also karyophilic and promotes productive infection of growth arrested cells in the absence of the other two PIC karyophilic proteins [6]. The significance of these karyophilic proteins has been exquisitely illustrated by demonstrating the absence of HIV infection due to the failure of nuclear entry of the viral genome in NLS-mutants of this virus [11]. The existence of redundant nuclear import activities in HIV-1 suggests that its karyophilic proteins may use different and distinct pathways. Indeed, these karyophilic proteins possess NLSs of different sequences and charges. The HIV-1 MA possesses an NLS with features analogous to the prototypic NLS of the SV40 large T antigen [3,11]. On the other hand, the NLS of Vpr is not yet fully characterized, and the nuclear import activity of this protein was attributed to either the negatively charged putative amphiphilic α-helix [12] located between residues 17–34 [13,14] or to the arginine-rich domain situated at the C-terminus of the protein [15]. The NLS of HIV-1 integrase has as yet not been determined [6].

In the present work we have used peptides derived from HIV-1 Vpr conjugated to labeled bovine serum albumin (BSA) and the in-vitro nuclear import assay system, namely permeabilized cultured cells, to better characterize the NLS region of Vpr. Permeabilized cultured animal cells have been extensively used as an in-vitro system to study various aspects related to nuclear import of karyophilic proteins, as well as the requirement of cellular proteins to promote such entry. In such assay systems, fluorescently [16,17] or biotin [18] labeled BSA molecules covalently conjugated to an NLS peptide are usually used as a transport substrate. The choice of such a protocol for labeling is not only due to the fact that most of the NLS-bearing proteins are not available in their purified form, but mainly because the labeling of such proteins may lead to inactivation of their nuclear translocation ability.

Here, we have shown that peptides derived from the N-terminal region putative helix of Vpr, and not from its C-terminus – both of which were suggested to mediate nuclear import of Vpr [13–15] – promoted entry of labeled BSA into nuclei of permeabilized cells.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PIC, preintegration complex; MA, matrix protein; NLS, nuclear localization signal; Vpr, viral protein r; BSA, bovine serum albumin; TFA, trifluoroacetic acid; TOF-MS, time of flight-mass spectrometry; TDW, triple distilled water; AAA, amino acid analysis; SV40, simian virus 40; WGA, wheat germ agglutinin; NPC, nuclear pore complex; N.D., not determined; NEM, *N*-ethyl maleimide

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Table 1
The ability of VprN, but not of VprC, to mediate nuclear import of BSA conjugates in permeabilized cells[a]

Conjugate:	VprN-BSA	VprC-BSA	SV40-NLS-BSA
Experimental conditions	Nuclear entry (%)[b]		
With cytosolic factors	100	0	100
Without cytosolic factors	100	0	15.2
4°C	25	N.D.	17
(−)ATP	26.7	N.D.	21.8
+WGA	53.6	N.D.	11.9
+NEM	46.7	N.D.	1.5

[a]Peptides bearing the sequences of residues 17–34 of HIV-1 Vpr protein (VprN), the C-terminal arginine rich domain of Vpr (residues 77–96, VprC), and the NLS of SV40 large T-antigen were synthesized and conjugated biotinylated BSA. The entry of the conjugates into nuclei of permeabilized Colo cells was estimated as described in Section 2. ATP was depleted as described [17]. WGA (0.6 mg/ml) and NEM (5 mM) were preincubated for 30 min at room temperature with the permeabilized cells before adding the BSA conjugates.
[b]Entry of each conjugate in the presence of cytosolic factors was considered as 100%.

2. Materials and methods

2.1. Cultured cells

Colo-205 (human colon adenocarcinoma cells (ATCC CCL 222)) were maintained in RPMI 1640 medium, supplemented with 10% FCS, 0.3 g/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Beit Haemek, Israel).

Monolayer cultures of HeLa cells were grown in DMEM growth medium supplemented with 10% FCS as previously described [17].

2.2. Synthesis of linear peptides

Linear NLS peptides were synthesized on Rink amide resin (loading 0.5 mmol/g) using the Applied Biosystems Peptide Synthesizer model 433A, by the FastMoc chemistry. The peptides were cleaved from the resin using trifluoroacetic acid (TFA) with 5% anisole as a scavenger, precipitated from cold ether, dissolved in 30% acetic acid and lyophilized. Crude peptides were analyzed by reverse-phase HPLC (C3 column, gradient of 5–60% acetonitrile/triple distilled water (TDW) containing 0.1% TFA, 35 min) and characterized by time of flight-mass spectrometry (TOF-MS) and amino acid analysis. The following peptides were synthesized: SV40 large T-antigen NLS (PKKKRKVC), SV40-NLS mutant (CGPK*N*KRKV), SV40-NLS reverse peptide (CVKRKKKPG), VprN (C^{16}NEWTLELLEELKNEAVRHF^{34}), VprN mutant (^{16}NE*A*TLELL*P*ELKN*P*AVRHF^{34}), VprC (C^{77}RHSRIGVTRQRRARNGASRS^{96}), HIV-MA-NLS (C^{25}GKKKYKLKH^{33}) and HIV-Nef-NLS (C^{7}KRSMGGWSAIRERMRR^{22}). Cysteine residues were added to the N-terminus or to the C-terminus of the original sequences to promote binding to BSA.

2.3. Estimation of nuclear import by fluorescence microscopy observations

HeLa cells were cultivated on 10-mm coverslips to a subconfluent density and then permeabilized with digitonin as described before [19] and slightly modified recently [17]. Peptides bearing the NLS of the SV40 large T-antigen, VprN, HIV-MA-NLS and VprC were covalently attached to rhodamine labeled BSA molecules essentially as described before [16] and translocation of the resulting fluorescently labeled NLS-BSA molecules (FL-NLS-BSA) into nuclei of digitonin-permeabilized HeLa cells was followed by fluorescent microscopy observations, as previously described [19].

2.4. Quantitative analysis of nuclear import in an in-vitro system

Nuclear import was quantitatively determined by the ELISA based method using biotin-labeled BSA as described before [18] and recently modified [20]. The results given are an average of triplicate ELISA determination, standard deviation of which never exceeded ±20%.

3. Results

3.1. The N-terminal helical region of Vpr, and not the C-terminal region, mediates nuclear uptake

Peptides derived from the N- and C-terminal regions of Vpr (designated VprN and VprC, see Table 1) were conjugated to rhodamine-BSA and their karyophilic properties were tested by fluorescent microscopy. As can be seen (Fig. 1), the VprN-BSA conjugates accumulated within the cell nuclei. In contrast, in an ATP-depleted system, at 4°C, as well as in the presence of wheat germ agglutinin (WGA) and of *N*-ethyl maleimide (NEM), VprN-BSA was retained in the cytoplasm, completely excluded from the nuclei (Fig. 1 and data not shown). The fluorescent microscopy observations were confirmed by a quantitative estimation of nuclear import using the ELISA-based method. As can be seen (Table 1), in ATP-depleted cells and at 4°C, nuclear import was reduced by about 75%, and in the presence of WGA and NEM by about 50% compared to the values obtained in the control system. In this regard, the observed VprN mediated nuclear import resembles in its features nuclear entry of SV40-NLS-BSA conjugates, which is considered to be the prototypic nuclear transport substrate (Table 1 and [16]). The requirement for ATP and the inhibition by WGA clearly suggest that the VprN-BSA conjugates are translocated via the nuclear pore complex (NPC) by a mechanism similar to that used by other karyophilic proteins. On the other hand, VprN-BSA entry into nuclei did not require the addition of soluble cytosolic extract, as was established by fluorescent microscopy studies (not shown) and by the ELISA based method (Table 1). Addition of cytosolic factors, which are lost during the permeabilization process, is essential for the reconstitution of the nuclear import machinery in digitonin-permeabilized cells as exemplified by using the SV40 mediated nuclear import (Table 1 and [16]). The ability of VprN to promote nuclear import of VprN-BSA in the absence of soluble factors may indicate that

Table 2
Influence of different peptides on nuclear import of fluorescently labeled VprN-BSA conjugates[a]

Peptide	Nuclear import of	
Conjugate:	VprN-BSA	SV40-NLS-BSA
VprN	+	+
VprN mutant	+	−
VprC	−	−
SV40-NLS	+/−	−
SV40-NLS mutant	+	+
SV40-NLS reverse	+	+
HIV-MA-NLS	−	−
HIV-Nef-NLS	−	−

[a]Synthetic peptide bearing the sequence of residues 17–34 of HIV-1 Vpr protein (VprN) was conjugated to rhodamine-BSA and the nuclear import of the conjugates was followed by fluorescent microscopy. For peptide sequences and estimation of nuclear import see Section 2. Peptides were added in a molar ratio (peptide/transport substrate) of 140:1.

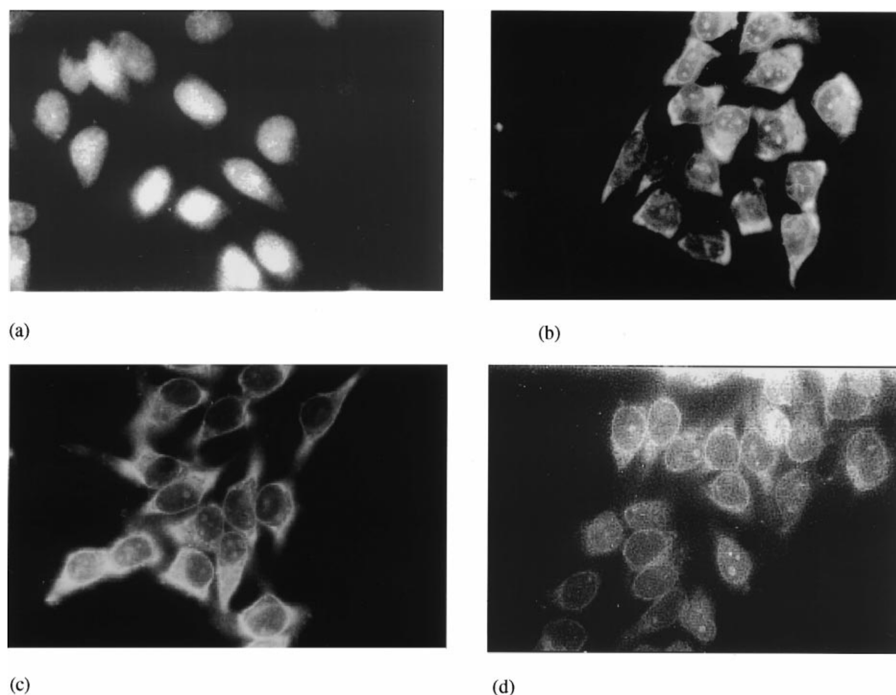

Fig. 1. Fluorescent microscopy observations of VprN-mediated nuclear uptake. The experimental conditions were as described in Section 2. Nuclear uptake of rhodamine-VprN-BSA was determined in the following systems. a: In the absence of cytosolic factors; b: at 4°C; c: with VprC (molar ratio of 200:1 to the transport substrate); d: with WGA (0.6 mg/ml).

Vpr uses a unique nuclear import pathway, as suggested before [5].

As opposed to the promotion of nuclear import activity by VprN, VprC did not show any karyophilic properties, and its conjugates with BSA were retained in the cytoplasm of the permeabilized cells (Table 1). However, VprC as well as peptides bearing the NLS sequences of the HIV proteins Nef and MA, strongly inhibited nuclear import of VprN-BSA (Table 2).

Since nuclear entry of VprN-BSA resembles in its features nuclear entry of other karyophilic proteins, it was anticipated that the free VprN peptide will efficiently block the entry of VprN-BSA. Inhibition of nuclear import of NLS-BSA conjugates by free NLS peptides has been shown before [16,20], and is used to demonstrate specific nuclear import of karyophilic proteins. Surprisingly, no inhibition was observed when the free VprN peptide was added to the nuclear import assay system. Fluorescent microscopy studies revealed that the fluorescent VprN-BSA molecules accumulated in the nuclei in the presence of VprN peptide (as well as in its absence) (Table 2). Also, the addition of the SV40-NLS peptide hardly inhibited nuclear entry of VprN-BSA, while it completely blocked nuclear entry of SV40-NLS-BSA (Table 2). No inhibition was observed either in the presence of the mutant SV40-NLS, the peptide bearing the reverse sequence of the SV40-NLS, and the mutant VprN (VprN W18A, E24P, E29P) (Table 2). It is noteworthy that recently it has been reported that SV40-NLS peptide did not inhibit the binding of Vpr to karyopherin-α [7].

3.2. *The effect of free VprN peptide on nuclear import in permeabilized cells: promotion of non-specific translocation*

As mentioned above (and see Table 2), the free VprN peptide did not inhibit nuclear entry either of VprN-BSA or of SV40-BSA conjugate. It was therefore of interest to investigate its effect on the entry of other NLS-BSA conjugates. Surprisingly, in the presence of VprN peptide, transport substrates such as SV40-NLS-BSA were found to accumulate within nuclei even in ATP depleted cells and/or in the absence of cytosolic factors, conditions in which, otherwise, such conjugates are exclusively retained in the cytoplasm (see also Table 1). A non-specific accumulation of SV40-NLS-BSA within the intranuclear space is, thus, observed in the presence of VprN peptide. This, indeed, was confirmed by the results (Fig. 2) showing that fluorescent BSA molecules lacking any conjugated NLS peptides, which by all means are not karyophilic and therefore should be retained in the cytoplasm, were found within the nuclei in the presence of VprN peptide. Furthermore, the addition of VprN peptide caused significant nuclear accumulation of HIV-MA-NLS-BSA conjugates (Fig. 2), nuclear entry of which is usually poor and almost undetected ([10] and unpublished results). Careful examination of the nuclei containing the fluorescently labeled molecules, which accumulated in the presence of VprN (Fig. 2a,c,d), shows that they retained their shape and that their nuclear envelope appears intact. Total disruption of the nuclear envelope by the VprN peptide seems unlikely, since it should result in an equal distribution of the dye within the whole microscopic field (as in the case following the addition of a detergent, such as Triton X-100), but not in accumulation of the fluorescent dye within the intranuclear space.

4. Discussion

The results of the present work clearly attribute the karyophilic properties of the HIV-1 Vpr to its putative α-helical region located between residues 17–34. This conclusion is

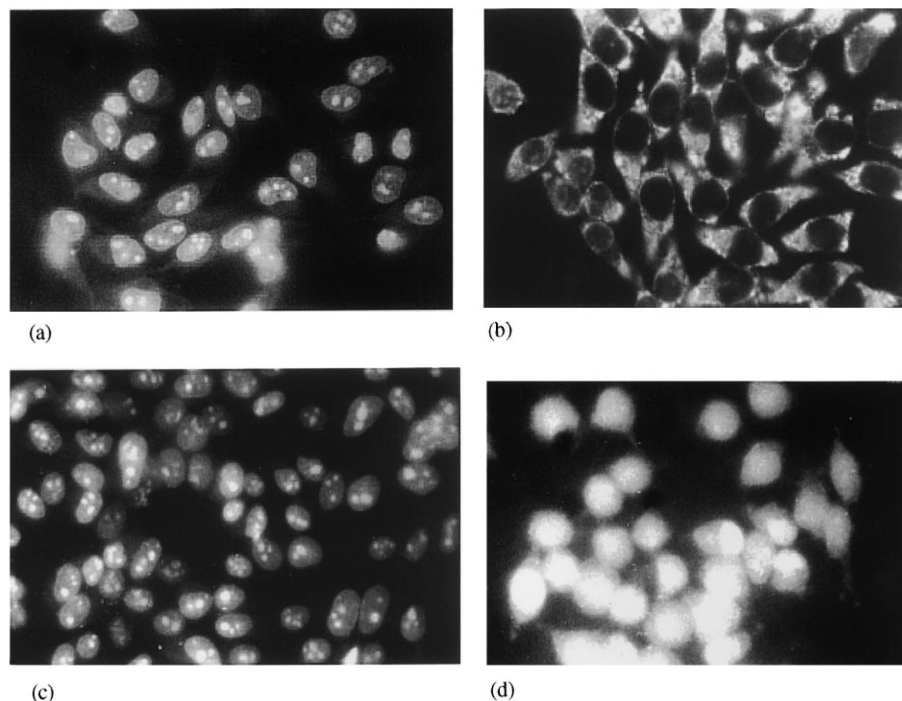

Fig. 2. Effect of free VprN on nuclear import. VprN peptide was added in a molar ratio of 200:1 to the following systems. a: Rhodamine-SV40-BSA without cytosolic factors and in the absence of ATP. b: The same as a but in the absence of VprN. c: MA-NLS-rhodamine-BSA. d: Rhodamine-BSA (without NLS).

based mainly on our observation showing that VprN was able to mediate nuclear entry of fluorescently labeled BSA. Although translocation of VprN-BSA conjugates into nuclei of permeabilized cells did not require the addition of cytosolic factors, it was ATP and temperature dependent and was inhibited by WGA and NEM, properties which characterize typical NLS-mediated nuclear import of karyophilic proteins. On the other hand, a peptide derived from the arginine-rich domain located between residues 77–96 at the C-terminus of Vpr (VprC), failed to show karyophilic properties when conjugated to BSA. VprC-BSA conjugates were retained in the cytoplasm of the permeabilized cells. Our present work strengthens previous suggestions, based on genetic studies using Vpr mutants [13,14], that the N-terminal putative α-helical region of Vpr confers the karyophilic properties of this protein. The present use of VprN-BSA conjugates enables us to limit the active region to residues 17–34 and to prove for the first time that this region, and not VprC, possesses an NLS activity. This region is negatively charged, as opposed to the positively charged consensus NLS sequences, such as that of SV40 T-antigen [21].

The unique properties of the Vpr-NLS region indicate that the Vpr protein uses a specific, yet uncharacterized, receptor, different from that used by the positively charged NLSs such as α-importin [22–24]. Indeed, this was suggested before [5], and may explain our results showing nuclear entry of VprN-BSA in the absence of cytosolic factors. Evidently, VprN (as well as Vpr) may interact with cellullar or nuclear envelope components which are retained within the permeabilized cells following the digitonin treatment. The result showing that SV40-NLS peptide hardly inhibited nuclear import of the VprN-BSA, as opposed to its ability to block nuclear import of SV40-NLS-BSA conjugates, strengthens our above assumption and fits previous studies showing that induction of nuclear entry of HIV-PIC by Vpr was not inhibited by SV40-NLS peptide [5].

Inhibition of nuclear entry of VprN-BSA conjugate by VprC, Nef-NLS and HIV-MA-NLS peptides may be explained by electrostatic interaction between these positively charged peptides and the negatively charged VprN. Since VprN and VprC are located within the same protein, an intramolecular interaction between these two regions can be proposed, suggesting a regulatory role for VprC. This will be clarified following future determination of the three-dimensional structure of Vpr.

Interestingly, addition of free VprN to the in-vitro nuclear import assay system promoted nuclear entry of fluorescently labeled conjugates which otherwise would have been retained in the cytoplasm. Nuclear presence of SV40-NLS-BSA conjugates in ATP depleted cells following the addition of free VprN peptide could have been explained by an electrostatic interaction between the positively charged NLS and the negatively charged DNA in the nuclei. The same sequence of events could have explained similar results obtained with HIV-MA-NLS-BSA conjugates, which under regular conditions and even in the presence of ATP and cytosolic factors remain in the cytoplasm (data not shown and see also [10]). However, the promotion of nuclear uptake of BSA molecules lacking any positively charged NLS, following free VprN peptide addition, cannot be explained by an electrostatic interaction with the DNA. Therefore, it is our view that the free VprN peptides do not act like a detergent and do not totally disrupt or solubilize the nuclear envelope. This view is supported by our microscopic observations showing preservation of the nuclear shape in the presence of VprN. In this regard, it should be mentioned that recently it has been shown that Vpr

induces the nuclear entry of the high molecular weight HIV-PIC [7] and therefore a regulatory effect on nuclear entry was attributed to Vpr. Our results indicate the same effect and pinpoint the regulatory activity to the NLS region of Vpr. VprN may either interact with karyophilic or non-karyophilic proteins and allow their entry into the nuclei, or alternatively interact with the NPC and increase its permeability or its pore size. Currently, studies in our laboratory are conducted to clarify these possibilities and to find out whether VprN interacts directly with the NPC.

During the preparation of the present manuscript, a paper has been published [25] showing unequivocally, by molecular biology methods, that only the nuclear transport activity of the HIV-1 Vpr, and not its other biological functions, is located within the putative amphipathic helical structure. These results strongly confirm the results of the present work.

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