# Direct activation of protein phosphatase-2A$_0$ by HIV-1 encoded protein complex NCp7:vpr

H.Y. Lim Tung[a,b,*], Hughes De Rocquigny[c], Ling-Jun Zhao[d], Xavier Cayla[a], Bernard P. Roques[c], René Ozon[a]

[a]*Laboratoire de Physiologie de la Reproduction, CNRS URA 1449, INRA, Université Pierre et Marie Curie, Bat B, 7 ème Etage, Case 13, 9 Quai Saint Bernard, 75005 Paris, France*
[b]*Protein and Peptide Research Laboratory, CFSR Biomedical Science Institute, Houston, TX 77068, USA*
[c]*Département de Pharmacochimie Moleculaire et Structurale, INSERM U266, CNRS URA D 1500, 75006 Paris, France*
[d]*Institute for Molecular Virology, School of Medicine, Saint Louis University Health Sciences Center, Saint Louis, MO 63110, USA*

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**Abstract The effects of HIV-1 encoded proteins NCp7, vpr and NCp7:vpr complex on the activity of protein phosphatase-2A$_0$ have been tested. We report that NCp7 is an activator of protein phosphatase-2A$_0$ and that vpr activated protein phosphatase-2A$_0$ only slightly. We also report that NCp7 and vpr form a tight complex which becomes a more potent activator of protein phosphatase-2A$_0$ than NCp7 alone. The ability of NCp7 to activate protein phosphatase-2A$_0$ is regulated by vpr. The C-terminal portion of vpr prevents NCp7 from activating protein phosphatase-2A$_0$ while the N-terminal portion of vpr potentiates the effect of NCp7 on the activity of protein phosphatase-2A$_0$. Our findings indicate that vpr may be acting as a targeting subunit which directs NCp7 to activate protein phosphatase-2A$_0$. In view of the fact that protein phosphatase-2A functions as an inhibitor of G$_0$ to M transition of the cell cycle and is involved in other key cellular processes such as the control of RNA transcription, the results presented in this report may explain how HIV-1 causes cell cycle arrest which may lead to CD$^{4+}$ T cell depletion and also how it disturbs normal cellular processes of its host cell.**

*Key words:* Protein phosphatase-2A; HIV-1; NCp7; vpr

## 1. Introduction

HIV-1 encoded nucleocapsid protein (NCp7) is a small basic protein with two zinc fingers of the $CX_2CX_4HX_4C$ type that are linked by a short basic sequence [1,2]. Mutations of key residues within the NCp7 molecule result in poorly infectious or non-infectious viruses [3,4]. NCp7 functions in RNA dimerization and packaging of the viral genomes, the annealing of replication primer tRNA onto the primer binding site (PBS) and the initiation of reverse transcription [1–5]. NCp7 may have other functions. It has been demonstrated that NCp7 interacts with another HIV-1 encoded protein termed vpr [6]. Several laboratories have shown that vpr causes cell cycle arrest at the G$_2$ to M transition in several cell types [7–13]. Because okadaic acid, a potent inhibitor of protein phosphatase-2A, can reverse the action of vpr, it is possible that vpr acts by modulating directly or indirectly the activity of protein phosphatase-2A which is an inhibitor of the G$_2$ to M transition of the cell cycle [8,14,15]. Both NCp7 and vpr have stretches of basic residues that are important for their functions and it also happens that protein phosphatase-2A$_0$ which has low basal activity requires polycations for full activity [3,4,16]. Protamine is an example of a highly basic protein which can act as an activator of protein phosphatase-2A in vitro [16]. The sequences of protamine, NCp7 and vpr resemble each other in terms of the clustering of the basic arginine residues [1,2,17,18]. We therefore tested whether NCp7, vpr and NCp7:vpr can act as activators of protein phosphatase-2A.

## 2. Materials and methods

### 2.1. Materials

ATP, benzamidine, poly-L-lysine agarose, protamine agarose, DEAE Sepharose, Sephacryl S-300 and protamine were purchased from Sigma. [$\gamma$-$^{32}$P]ATP was purchased from New England Nuclear. Protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (ABF) was purchased from Interchim (France). All the other reagents were of the highest grade.

### 2.2. Protein preparations

$^{32}$P-labeled phosphorylase a was prepared by phosphorylation of phosphorylase b with phosphorylase kinase as described in [19]. HIV-1 encoded protein NCp7, the N-terminal portion of vpr (vpr1–51) and the C-terminal portion of vpr (vpr52–96) were prepared by chemical synthesis on an automated Applied Biosystems Solid Phase Peptide Synthesizer as described previously [20]. Full length vpr was prepared by overexpression in bacteria as described previously [21]. HIV-1 encoded protein complex NCp7:vpr was prepared by incubating 15 μg of NCp7 and 15 μg of vpr in 50 mM imidazole-Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol for 30 min at 30°C. The incubation mixture was then gel filtered through a Superose 12 column equilibrated in incubation buffer plus 0.2 M NaCl. Fractions containing NCp7:vpr complex were analyzed by SDS-PAGE, pooled and concentrated by vacuum dialysis.

### 2.3. Measurement of protein concentrations

Protein was determined by the method of Bradford [22].

### 2.4. Assay of protein phosphatase-2A$_0$

The assay of protein phosphatase-2A$_0$ consisted of 0.02 ml of enzyme solution in 50 mM imidazole-Cl pH 7.3, 0.2 mM EGTA, 1 mg/ml bovine serum albumin and 0.1% (v/v) 2-mercaptoethanol (assay buffer), 0.02 ml of protamine at 10 μg/ml in assay buffer and 0.02 ml of $^{32}$P-labeled phosphorylase a at 3 mg/ml in assay buffer containing 15 mM caffeine. The assay components were preincubated for 10 min prior to initiating the reaction with [$^{32}$P]phosphorylase a. One unit of protein phosphatase is the amount of enzyme that catalyzes the release of 1 nmol of phosphate from phosphorylase a per minute [19].

When testing the effects of NCp7, vpr and NCp7:vpr complex, these molecules were added instead of protamine and incubated as described above.

### 2.5. SDS-PAGE

SDS-PAGE was performed as described in [23].

*Corresponding author. Fax: 33 (1) 44-27-34-72.
E-mail: ltung@hall.snv.jussieu.fr


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*2.6. Purification of protein phosphatase-2A$_0$ from pig brain*

Pig brains (2 kg) from freshly killed animals were obtained from the local slaughterhouse, cut into small pieces and homogenized in a Waring blender in 2 volumes of 50 mM imidazole-Cl pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and the proteinase inhibitors benzamidine (1 mM) and ABF (0.1 mM). All operations were performed in the cold room. The homogenate was centrifuged for 30 min at 6000×*g* and the supernatant was collected and filtered through glass wool. The filtrate was applied to a DEAE Sepharose (10×15 cm) equilibrated with buffer A (25 mM imidazole-Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine and 0.1 mM ABF. The column was washed with 10 liters of buffer A plus 0.025 mM NaCl and then eluted with buffer A plus 0.3 M NaCl. The active fractions were collected and concentrated by 60% ammonium sulfate saturation. The concentrate was dialyzed extensively overnight against buffer A plus 10% (v/v) glycerol (buffer B). The dialysate was centrifuged at 100 000×*g* for 1 h and loaded onto a DEAE Sepharose column (2.5×50 cm) equilibrated in buffer B. The column was washed with buffer B plus 0.025 M NaCl and then developed with a 1000 ml linear gradient of buffer B plus 0.025 M NaCl to buffer B plus 0.4 M NaCl. Two major peaks of protamine stimulated protein phosphatase activity were detected, the first eluting at 0.13 M NaCl and the second at 0.22 M NaCl. We established that the first peak is protein phosphatase-2A$_0$ and the second peak is protein phosphatase-2A$_1$. The peak corresponding to protein phosphatase-2A$_0$ was pooled, diluted 3-fold in buffer B and then loaded onto a poly-L-lysine agarose column (1.5×30) equilibrated in buffer B. The column was washed with buffer B plus 0.15 M NaCl and then eluted with a linear gradient of buffer B plus 0.15 M NaCl to buffer B plus 0.6 M NaCl. The active fractions eluting at around 0.25 M NaCl were pooled, diluted 2-fold in buffer B and loaded onto a protamine agarose column (1.5×20 cm) equilibrated in buffer B. The column was washed with buffer B plus 0.4 M NaCl and eluted with a 400 ml gradient of buffer B plus 0.4 M NaCl to buffer B plus 1.0 M NaCl. The active fractions eluting at around 0.65 M NaCl were pooled, concentrated by vacuum dialysis to about 4 ml and loaded onto a Sephacryl S-300 column (2.5×88) equilibrated in 50 mM imidazole-Cl pH 7.2, 0.2 mM EGTA, 0.2 M NaCl, 10% (v/v) glycerol, 1 mM benzamidine and 0.1 mM ABS. The active fractions were collected, concentrated by vacuum dialysis, dialysed against buffer A plus 50% (v/v) glycerol and stored as such at −20°C. Protein phosphatase-2A$_0$ remained active for several months.

## 3. Results

Protein phosphatase-2A is one of the four major types of protein phosphatases in the cell [16,24]. Many subtypes of protein phosphatase-2A exist in the cell and these have been termed protein phosphatase-2A$_0$, protein phosphatase-2A$_1$, protein phosphatase-2A$_2$ and protein phosphatase-2A$_M$ [16,24–26]. To test whether NCp7 or vpr can act as activators of protein phosphatase-2A, it is important that our preparation of protein phosphatase-2A has a low basal activity. Protein phosphatase-2A$_0$ has very low basal activity compared to the other subtypes [16,24]. A survey of the relative activity and abundance of protein phosphatase-2A in various tissues indicated that brain tissue has a large amount of protein phosphatase-2A that has essentially low specific activity (unpublished data). We therefore chose to purify protein phosphatase-2A$_0$ from pig brain by successive chromatographies of a pig brain extract on DEAE Sepharose (batch elution), DEAE Sepharose (gradient elution), poly-L-lysine agarose, protamine agarose and Sephacryl S-300. The purification of protein phosphatase from pig brain is summarized in Table 1 and Fig. 1. The preparation of pig brain protein phosphatase-2A$_0$ exhibited four major proteins of apparent molecular masses 60 kDa, 53 kDa, 48 kDa and 36 kDa that were in stoichiometric amounts as well as a number of more minor bands. The 60 kDa protein represents the A subunit, the 53 kDa the B′ subunit and the 36 kDa the C catalytic subunit [16,24]. We could not ascertain the identity of the 48 kDa protein. It may represent a novel subunit of protein phosphatase-2A$_0$ which functions as an inhibitor of the catalytic subunit (manuscript in preparation). We have tentatively termed it the D subunit awaiting further analysis of its primary structure. As expected, the specific activity of the enzyme was about 110 units per mg protein, an order of magnitude lower than those from other tissues [16,24,25]. The enzyme preparation is therefore ideal for testing our hypothesis.

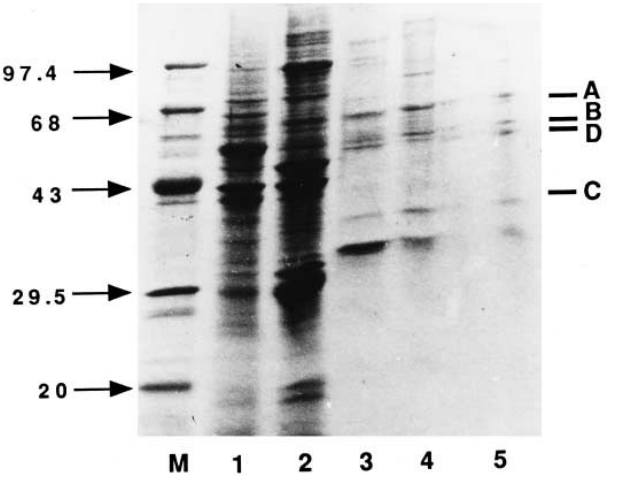


Fig. 1. SDS-PAGE pattern of pooled active fractions at each step of purification of protein phosphatase-2A$_0$. The gel was stained with Coomassie blue. Lane M, protein standards, from top to bottom: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and bovine α-lactalbumin; lane 1, first DEAE Sepharose step; lane 2, second DEAE Sepharose step; lane 3, poly-L-lysine agarose step; lane 4, protamine agarose step; lane 5, Sephacryl S-300 step. The bars on the right indicate the migration positions of the 60 kDa A subunit, the 53 kDa B′ subunit, the 48 kDa D subunit and the 36 kDa C subunit. The C subunit is the catalytic subunit whereas the other subunits are regulatory subunits.

Table 1
Purification of protein phosphatase-2A$_0$ from pig brain

| | Step | Volume (ml) | Protein (mg) | Activity (units) | Specific activity (units/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1. | First DEAE Sepharose | 260 | 13 000 | 1945 | 0.15 | 1 | 100 |
| 2. | Second DEAE Sepharose | 150 | 450 | 827 | 1.84 | 12 | 43 |
| 3. | Poly-L-lysine agarose | 67 | 44 | 523 | 11.89 | 79 | 27 |
| 4. | Protamine agarose | 50 | 5.60 | 478 | 85.36 | 569 | 25 |
| 5. | Sephacryl S-300 | 10 | 0.85 | 94 | 110.59 | 734 | 5 |

2 kg of pig brain was obtained from freshly killed animals from the local slaughterhouse and homogenized in two volumes of homogenization buffer as described in Section 2.

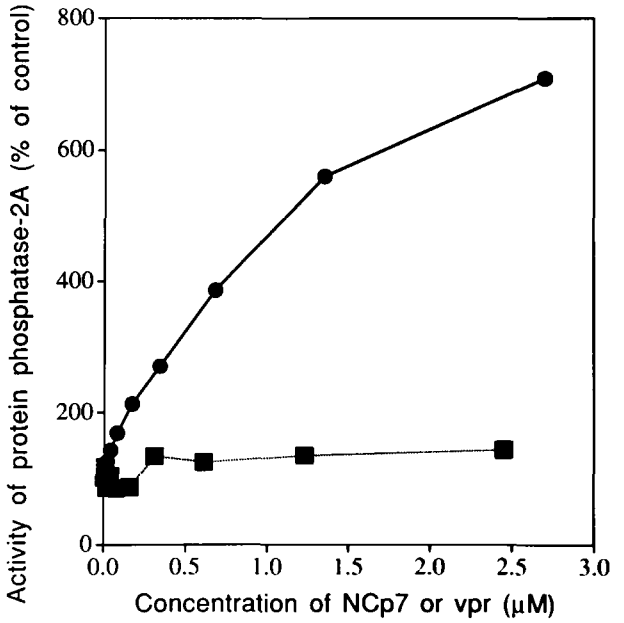


Fig. 2. Effects of HIV-1 encoded proteins NCp7 and vpr on the activity of protein phosphatase-$2A_0$. NCp7 was prepared and purified as described in [29]. The protein vpr was overexpressed in bacteria and purified as described in [30]. Protein phosphatase-$2A_0$ was assayed as described in [21] in the presence of various concentrations of NCp7 (circles) or vpr (squares) as indicated. 100% protein phosphatase-$2A_0$ activity is equivalent to 0.02 unit.

As shown in Fig. 2, NCp7 was a better activator of protein phosphatase-$2A_0$ than vpr with half-maximal activation occurring at about 0.45 μM and 0.25 μM for NCp7 and vpr respectively. NCp7 activated protein phosphatase-$2A_0$ by almost 7-fold whereas vpr activated protein phosphatase-$2A_0$ by only 1.5-fold. NCp7 has been reported to interact with the full length vpr [6]. We therefore prepared NCp7:vpr complex by incubation of the two proteins at 30°C as described in the legend to Fig. 3 which shows that the NCp7:vpr complex can activate protein phosphatase-$2A_0$ with half-maximal activation occurring at around 0.14 μM. Maximal activation of protein phosphatase-$2A_0$ was about 12-fold and occurred at around 0.3 μM. The NCp7:vpr complex is therefore a much more potent activator of protein phosphatase-$2A_0$ than NCp7 alone. These data collectively indicate that vpr modulates the ability of NCp7 to activate protein phosphatase-$2A_0$ by lowering its $K_a$ for the enzyme. However, at high concentration (above 0.3 μM), the activation of the enzyme by the NCp7:vpr complex was reversed. This dual effect of the NCp7:vpr complex is also observed with the other activators of protein phosphatase-2A and also in the case of the regulation of protein phosphatase-$1_I$ by its regulatory protein [16,24,27]. The dual effect of a protein regulator on a protein phosphatase can be explained by the presence of more than one binding site on the enzyme for the regulator protein. The high affinity binding site would be responsible for activation while the low affinity binding site would be responsible for inactivation. As shown in Fig. 4, at a concentration of above 1 μM, the C-terminal domain of vpr acts to prevent the activation of protein phosphatase-$2A_0$ by NCp7 while the N-terminal domain of vpr does not prevent but potentiates the activation of protein phosphatase-$2A_0$ by NCp7. The N- and C-terminal domains of vpr do not by themselves activate protein phosphatase-$2A_0$ significantly (data not shown). The concentration of protein phosphatase-$2A_0$ is estimated to be around 1 μM in the cell and its activation by NCp7:vpr complex occurs at sub-μM concentration. The effect of NCp7:vpr complex on the activity of protein phosphatase-$2A_0$ is therefore not a substrate directed effect since the concentration of the substrate in the assay is over 10 μM [19].

## 4. Discussion

This is the first report describing the activation of protein phosphatase-$2A_0$ by two HIV-1 encoded proteins NCp7 and vpr. The significance of this work is that protein phosphatase-2A is activated by a complex of the viral proteins which may occur at concentrations that prevail in the cell. Protein phosphatase-2A is an inhibitor of the $G_2$ to M transition of the cell cycle and it is also involved in the control of RNA transcription [14,15,28,29]. Our work shows that vpr does not by itself significantly activate protein phosphatase-2A (at the most, there was an activation of 1.5-fold). However, it is able to modulate the activity of protein phosphatase-2A because of its ability to form a tight complex with another HIV-1 encoded protein, NCp7. Vpr may therefore be acting as a targeting unit that directs NCp7 to activate protein phosphatase-2A. The finding that protein phosphatase-$2A_0$ is activated by NCp7:vpr complex is consistent with the proposed roles of these two proteins in HIV-1 physiology and pathophysiology.

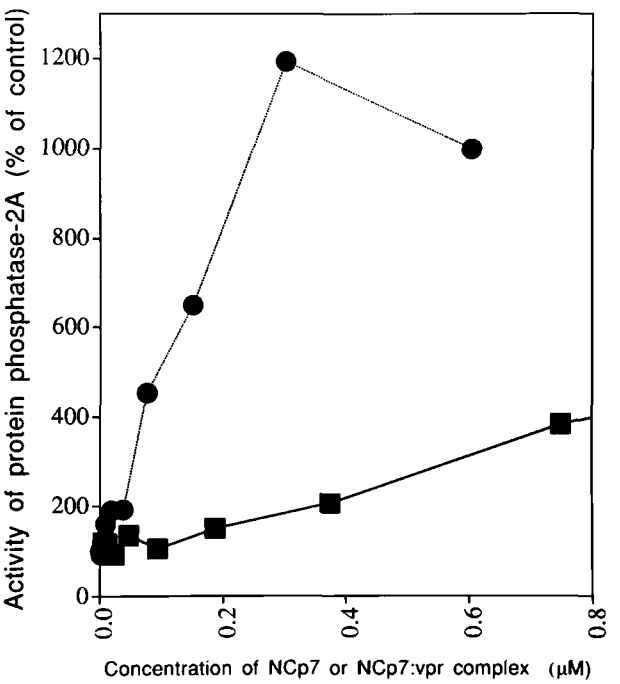


Fig. 3. Effect of HIV-1 encoded protein complex NCp7:vpr on the activity of protein phosphatase-$2A_0$. 15 μg of NCp7 and 15 μg of vpr were incubated together in 50 mM imidazole-Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol for 30 min at 30°C. The complex of NCp7:vpr was then isolated by gel filtration through a Superose-12 column, concentrated by vacuum dialysis and used in the protein phosphatase-$2A_0$ assay. NCp7:vpr complex elutes as a species of apparent molecular mass 110 kDa on Superose 12. This value was used to calculate the molar concentration of NCp7:vpr complex. Protein phosphatase-$2A_0$ was assayed as described in [21] in the presence of various concentrations of NCp7:vpr complex (circles) or NCp7 alone (squares).

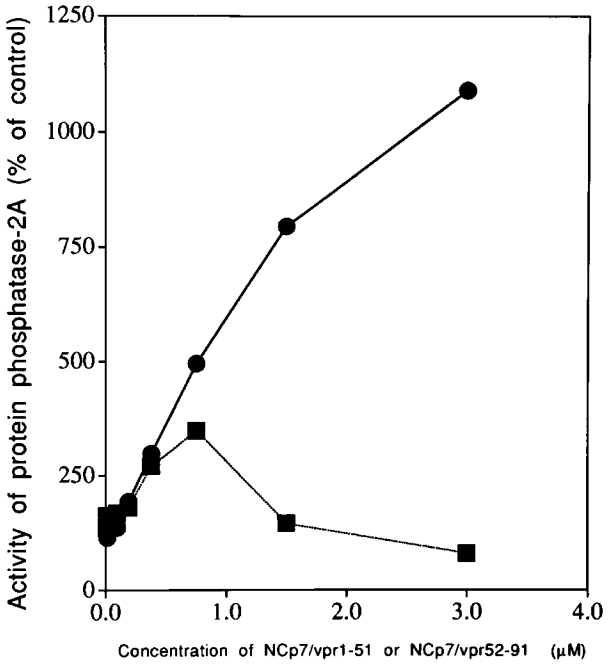


Fig. 4. Effect of HIV-1 encoded protein NCp7 on the activity of protein phosphatase-$2A_0$ in the presence of the N-terminal and C-terminal portions of vpr. NCp7 was preincubated for 10 min with equimolar amounts of the N-terminal portion of vpr (vpr-1-51) (circles) or the C-terminal portion of vpr (vpr52–96) (squares) and then mixed with protein phosphatase-$2A_0$. Following an incubation time of 10 min, the activity of protein phosphatase-$2A_0$ was measured as described in [21]. vpr1–51 and vpr52–96 were prepared by chemical synthesis on an automated solid phase Applied Biosystems peptide synthesizer.

It explains how HIV-1 causes cell cycle arrest at the $G_2$ to M transition in its host cells which may account for the severe depletion of $CD^{4+}$ T cells seen in the majority of HIV-1 infected individuals. It has been suggested that HIV-1 induced cell cycle arrest of activated $CD^{4+}$ T lymphocytes would result in non-clonal expansion of the infected cells [8]. The consequence of stopping the clonal expansion of certain $CD^{4+}$ T cells could be a cascade of events that may have catastrophic effects on cellular processes that control the proliferation of these cells and also of other cells that depend on factors that are produced by the infected cells. Our results may also explain how HIV-1 upregulates viral replication. Dephosphorylation of phosphorylation dependent transcription factors that bind to negative regulatory elements on the LTR by protein phosphatase-2A would result in upregulation of viral RNA transcription. Alternatively, dephosphorylation of dephosphorylation dependent transcription factors that bind to positive regulatory elements on the LTR by protein phosphatase-2A would also result in upregulation of viral RNA transcription [28,29]. The interaction of viral proteins with protein phosphatase-2A may underlie the mechanism by which various viruses interact and interfere with their hosts' normal cellular processes. In addition to HIV-1, other viruses such as simian virus 40, polyoma virus and adenovirus also interfere with their hosts' cellular machinery by targetting one of the subunits of protein phosphatase-2A and/or changing the specificity of the enzyme [31,32].

The finding that HIV-1 encoded protein complex NCp7:vpr is an activator of protein phosphatase-$2A_0$ is of significance because although the different subtypes of protein phosphatase-2A were identified many years ago and are known to be involved in the control of many key cellular reactions that may be linked to carcinogenesis and HIV-1 pathophysiology, the mechanisms that underlie these involvements have remained obscure [8,33]. The principal reason has been that no physiological regulators that may activate these latent enzymes could be identified. The present work and those demonstrating that vpr alone can cause cell cycle arrest at the $G_2$ to M transition would suggest that a molecular equivalent of NCp7 may be present in the cell to regulate the activities of the different subtypes of protein phosphatase-2A.

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