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The Vpr Protein of Human Immunodeficiency Virus Type 1 Binds to Nucleocapsid Protein p7 *in Vitro*

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The Vpr protein of human immunodeficiency virus type 1 (HIV-1) is incorporated into the virion by the Gag polyprotein precursor $Pr55^{gag}$. The importance of the $p6^{gag}$ sequence at the C-terminal end of $Pr55^{gag}$ has a crucial role in Vpr incorporation. To identify the Gag sequences directly involved in Vpr binding, we compared the Vpr binding affinities of the 71 amino acid nucleocapsid protein p7, the C-terminal peptide (35–71) p7C and $p6^{gag}$ by affinity chromatography. p7 and p7C have the strongest Vpr binding activities compared to $p6^{gag}$. These results suggest that the nucleocapsid protein and its C-terminal domain may be important for the incorporation of Vpr into the mature HIV-1 virion and the subsequent localisation of viral nucleic acid to the cell nucleus by Vpr.

The accessory protein Vpr is encoded by most primate immunodeficiency virus genomes (1). Vpr is the only accessory protein to be found in HIV-1 particles (2,3). Further studies have shown that the incorporation of Vpr into the HIV-1 virion requires the Gag polyprotein precursor $Pr55^{gag}$ (4). Vpr has been detected beneath the viral envelope in HIV-1 particles by immunoelectron microscopy (5), although to which HIV protein it binds could not be confirmed. Subsequently, HIV-1 Vpr influences nuclear localisation of viral nucleic acids in macrophage cells (6). Important sequences for the incorporation of Vpr into the HIV-1 virion have been localised to the $p6^{gag}$ region of $Pr55^{gag}$ (7) and more recently to a conserved motif near the C-terminus of p6 (8,9). $Pr55^{gag}$ is cleaved by the viral protease to yield the processed viral Gag structural proteins: the matrix protein p17 derived from the N-terminus, the major core protein p24 and nucleocapsid protein (NCp) p15 derived from the C-terminus of the $Pr55^{gag}$ (10). NCp15 is further processed to generate the mature nucleocapsid protein p7 and p6 (11). p7 contains two zinc binding domains which are essential for biological activity (12,13). Until now, there has been no demonstration of a direct binding by Vpr to any of the processed Gag proteins. In the present study, using affinity immobilisation of protein on peptide coupled to agarose and subsequent elution we demonstrate that Vpr binds to p7.

MATERIALS AND METHODS

Synthetic peptides. The p7 and p6 sequences used in this study were chosen from the LAI isolate of HIV-1 (14). The 52 amino acid p6, 71 amino acid p7 and C-terminal 37-residue [35–71] p7C peptide of p7 containing the second zinc binding domain (13) were chemically synthesised on a Model 431A Automated Peptide Synthesiser (Applied Biosystems) and purified by reversed-phase HPLC with acetonitrile-water gradients (to be published elsewhere). The molecular weights of the purified peptides were checked by mass spectrometry, using matrix-assisted laser desorption for p6 and p7C (School of Pharmacy, London, U.K.) and the electrospray technique for p7 (ETH, Zürich, Switzerland). The zinc-bound form of p7 or p7C was prepared by dissolving the peptides in 10 mM MOPS (Sigma) pH 7.0 containing 1 mM $ZnCl_2$ (5-molar equivalents of Zn^{++} to p7) and purged with Argon to prevent oxidation.

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Preparation of affinity gels. Affi-gel 10 and 15 are *N*-hydroxysuccinimide ester derivatives of agarose, and will conjugate proteins at neutral pH with isoelectric points (pI) of 6.5 to 11 (p7) and acidic proteins (p6), respectively (15). Zinc bound p7 and p7C, and lysozyme (Sigma) were coupled with Affi-gel 10 in zinc binding buffer. p6 and bovine serum albumin (BSA) (Sigma) were coupled to Affi-gel 15 in 10 mM MOPS buffer pH 7.0. For each peptide, 5 mg of protein was mixed per ml of gel in a total volume of 2 ml MOPS buffer by rotation for 16 hours at 4°C. Incorporation of the peptide was monitored by reading the optical density (280 nm) of the supernatant from the coupling reaction. Excess proteins and zinc in the supernatant were removed by washing in 10 mM MOPS, and the coupled and uncoupled gels (negative controls) were incubated with 1 M ethanolamine pH 8.0 to block non-specific binding sites (15).

Vpr expression. The DNA fragment encoding Vpr of the HIV LAI isolate sequence (1) was obtained by PCR and subcloned into baculovirus expression vector pVL1393 Pk (Invitrogen). The Vpr was overexpressed in *Spodopera frugiperda (Sf*9) cells as a fusion protein with a 14 amino acid tag at the C-terminus for detection of the recombinant Vpr with SV5-P-k antibody (16).

Vpr binding assay. Identical volumes of Vpr fusion protein-containing *Sf*9 cell extract (1% NP-40, 150 mM NaCl and 20 mM Tris pH 7.5) were loaded onto fixed volumes of the coupled or uncoupled gel in each case. The gel was washed with 1 × phosphate buffered saline (PBS pH 7.4). The proteins bound to the ligand on the gels were released by mixing the gel with 1 volume of 2 × SDS-PAGE loading buffer (65 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT and 2.5% bromophenol blue) and heated at 100°C for 4 minutes. The cell extract flowthrough (E), the last fraction of wash (L) and the protein released in SDS-PAGE loading buffer (B) were collected for Western blot analysis. To detect the Vpr present, collected samples were separated by SDS-PAGE on 10% acrylamide gels and transferred to nitrocellulose membrane (Hybond-C, Amersham) by electroblotting using a Sartoblot II-S apparatus (Sartorius) following the manufacturer's instructions. Blots were incubated with a solution containing 1 × PBS, 0.02% sodium azide and 5% fat free milk at 4°C for 16 hours to block non-specific binding sites. After washing with 4 × 250 ml of 1 × PBS, the blots were applied to two successive antibody binding reactions: with SV5-P-k-tag monoclonal antibody which can recognise the 14 amino acid tag (16) in the Vpr fusion protein followed by HRP-conjugated anti-mouse IgG (Amersham). Washing was also carried out between and after the two antibody binding reactions. The specific binding of antibodies on the blots was detected using an ECL detection system (Amersham) and the results were exposed on Hyperfilm (Amersham) and are shown in Fig. 1. Two negative controls were also set up with uncoupled Affi-gel 15 and 10, which were treated under identical conditions in parallel with p6, BSA, p7 and lysozyme.

RESULTS AND DISCUSSION

In Fig. 1 strong bands were present in all E sample lanes for the six binding experiment groups and in the Vpr cell extract. No signal was visible in the last fractions of wash collected from the six binding reactions (Fig. 1, L lanes). This result confirmed that the washing was sufficient for the

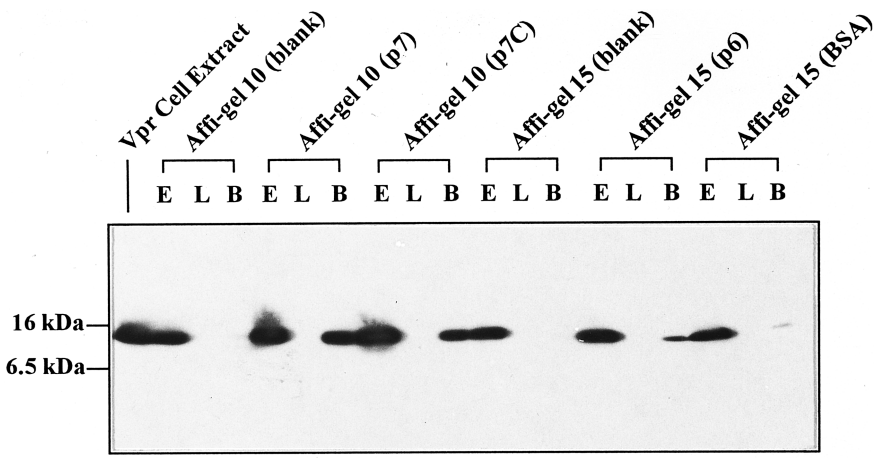

FIG. 1. Western blot analysis of samples collected from affinity chromatography. Cell extract flowthrough (E), last fraction of wash (L) and elution in SDS-PAGE loading buffer (B) were collected from uncoupled Affi-gel 10 [Affi-gel 10 (blank)], p7 coupled Affi-gel 10 [Affi-gel 10 (p7)], p7C coupled Affi-gel 10 [Affi-gel 10 (p7)], uncoupled Affi-gel 15 [Affi-gel 15 (blank)], p6 coupled Affi-gel 15 [Affi-gel 15 (p6)], and BSA coupled Affi-gel 15 [Affi-gel 15 (BSA)]. Samples E, L and B from each experiment were loaded in adjacent lanes for SDS-PAGE and examined by Western blot using SV5-P-k-tag antibody to recognise the Vpr fusion protein with the ECL (Amersham) detection system. A Vpr cell extract sample and protein size markers were also run alongside.

six binding experiments and any Vpr left in the gel should remain bound to the ligand. Inspection of the sample lanes B for both Affi-gel 10 (p7) and (p7C) (Fig. 1) showed a strong binding activity of Vpr with p7 and p7 C-terminal peptide. p6 coupled Affi-gel 15 showed a relatively low Vpr binding strength [see lane Affi-gel 15 (p6)-B]. As a negative control for Vpr binding reaction with p7 and p7C coupled on Affi-gel 10, uncoupled Affi-gel 10 showed no binding reaction with Vpr [see lane Affi-gel 10 (blank)-B]. p7 and p7C have pI values above 10 and consequently will be positively charged under the binding conditions used here. To exclude the possibility of purely electrostatic binding between Vpr and p7 or p7C we showed that lysozyme (pI 10.5) coupled to Affi-gel did not bind Vpr any stronger than Affi-gel 10 alone (data not shown). As a non-specific protein control on Affi-gel 15, BSA coupled to Affi-gel 15 showed little binding capacity with Vpr, which was demonstrated to be the same as the negative control of uncoupled Affi-gel 15 with Vpr [see lane Affi-gel 15 (blank)-B)].

It has been demonstrated that incorporation of Vpr into HIV virions requires the expression of *gag* products and is independent of *pol* and *env* expression (7). Direct interaction of Vpr with the $Pr55^{gag}$ was also demonstrated by immunoprecipitation (4). Here, we provide the first evidence for p7-Vpr binding. Other studies have shown that p6 plays an important role in Vpr incorporation (7–9). It has also demonstrated that Vpr can be incorporated into MLV when HIV p6 is fused to the C-termini of MLV *gag* gene products, while there is no Vpr incorporation when HIV p6 is not present in MLV *gag* products (8). In that study, the importance of HIV p6 for Vpr incorporation was stressed. It is worth noticing that MLV *gag* gene products contain NCp which has sequence homology with HIV p7 in the zinc binding domains (12,17), but has no equivalent protein with sequence homology to HIV p6 (14,18). It was recently shown that the conformation of the p7 sequence differs between p7 and p15, suggesting that the p6 sequence is important for p7 folding in the $Pr55^{gag}$ and NCp15 precursors (19). In our study, p6 showed very little binding capacity with Vpr compared to p7-Vpr binding. Combining our results and previous studies, it indicates that *in vivo* p7 may play an important role in the incorporation of Vpr into virions but p6 joined at the C-terminus of p7 in $Pr55^{gag}$ may be a crucial factor to enable p7 to form an accessible conformation for Vpr binding. The Vpr may remain bound to p7 in the mature virion following proteolytic cleavage from $Pr55^{gag}$. Additional support for the importance of p7 in Vpr binding was shown from a partial p7 deletion mutant of HIV, which resulted in impairment of Vpr incorporation into HIV particles (9). Also, a predicted α-helix in the N-terminal region of Vpr was shown to be important for incorporation into HIV by site-directed mutagenesis (20–22). This α-helix would have five glutamic acid residues on one side, and hydrophobic residues on the other. Substitution of one of the glutamic acids for lysine impaired Vpr incorporation (22), indicating the importance of negatively charged residues for binding with $Pr55^{gag}$. The most favorable location on $Pr55^{gag}$ for binding this α-helical motif would be expected to contain the highest concentration of positively charged residues, and this is the p7 sequence.

p7 has two zinc fingers which can be important for protein-protein interactions. An example is RelA, which directly binds to the zinc finger region of Sp1 and results in cooperative DNA binding to kappa B and Sp1 sites in the HIV-1 long terminal repeat (23). The glucocorticoid receptor is a zinc finger protein and was shown to be a target for the Vpr protein (24). It would be interesting to know whether Vpr-p7 binding takes place through the zinc fingers of p7. In this regard, p7C peptide which contains only the second zinc finger domain showed Vpr binding activity [see lanes for Affi-gel 10 (p7C), Fig. 1.]. It is known that the p7 remains associated with viral DNA after reverse transcription (25). Furthermore, the viral protease is known to be crucial for the integration of viral DNA by cleaving the first zinc finger domain of p7 leaving the second zinc finger intact as an active protein in this process (26) and which still may bind Vpr according to our *in vitro* studies. The p7-Vpr binding evidenced by this study suggests a possible role of p7 in the nuclear

localisation of viral DNA by Vpr. To confirm the p7 binding Vpr *in vivo* and in nuclear localisation, further binding studies are required using mutants of Vpr and NCp15.

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