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HIV-1 p6—Another viral interaction partner to the host cellular protein cyclophilin A

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ABSTRACT

The 52-amino acid human immunodeficiency virus type 1 (HIV-1) p6 protein has previously been recognized as a docking site for several cellular and viral binding factors and is important for the formation of infectious viruses. A particular structural feature of p6 is the notably high relative content of proline residues, located at positions 5, 7, 10, 11, 24, 30, 37 and 49 in the sequence. Proline *cis*/*trans* isomerism was detected for all these proline residues to such an extent that more than 40% of all p6 molecules contain at least one proline in a *cis* conformation. 2D ^{1}H nuclear magnetic resonance analysis of full-length HIV-1 p6 and p6 peptides established that cyclophilin A (CypA) interacts as a peptidyl–prolyl *cis*/*trans* isomerase with all proline residues of p6. Only catalytic amounts of CypA were necessary for the interaction with p6 to occur, strongly suggesting that the observed interaction is highly relevant *in vivo*. In addition, surface plasmon resonance studies revealed binding of full-length p6 to CypA, and that this binding was significantly stronger than any of its N- or C-terminal peptides. This study demonstrates the first identification of an interaction between HIV-1 p6 and the host cellular protein CypA. The mode of interaction involves both transient enzyme–substrate interactions and a more stable binding. The binding motifs of p6 to Tsg-101, ALIX and Vpr coincide with binding regions and catalytic sites of p6 to CypA, suggesting a potential role of CypA in modulating functional interactions of HIV-1.

1. Background

The human immunodeficiency virus type 1 (HIV-1) p6 protein is derived from the C-terminal of the Gag precursor protein Pr55gag, which is one of the three polyproteins that comprises the main structural components that are essential and sufficient for the formation of virus like particles (VLPs). HIV-1 p6 serves manifold functions as docking site for several cellular factors and fulfils important roles in the formation of infectious virus particles. p6 facilitates virus budding [1,2] and is required for the incorporation of the accessory protein viral protein R (Vpr) into virus particles [3–6]. In addition, HIV-1 p6 was recently shown to regulate capsid (CA) processing and virus core assembly [7] and found to be involved in regulating defective ribosomal product (DRiP) rate and thus MHC Class I antigen presentation of Gag [8]. Previously we demonstrated p6 adopts a helix-flexible helix structure under hydrophobic solution conditions; a short helix-1 (residues 14–18) is connected to a pronounced helix-2 (residues 33–44) by a flexible hinge region [9], which has been further supported by recent theoretical calculations [10]. The secondary structure of the protein is weaker in an aqueous solution [9].

The host cellular protein cyclophilin A (CypA) is a member of the protein family called cyclophilins that have a common ability to act as peptidyl–prolyl *cis*/*trans*-isomerases (PPIases). In addition to its PPIase activity CypA is suggested to act as a molecular chaperone to assist protein folding, assembly, and transportation processes [11]. CypA is found incorporated in newly budding particles of HIV-1 and represent a potential key molecule in future antiretroviral therapy since inhibitors of CypA, such as Cyclosporin A (CsA), suppresses HIV-1 replication [12–17]. CypA is found to interact with both HIV-1 CA and Vpr. Although these interactions have been extensively studied, the biological significance of CypA in the viral life cycle of HIV-1 is still not completely understood [17–22].

Recently, we discovered that catalytic sub-stoichiometric amounts of CypA catalyze prolyl *cis*/*trans* isomerization of all proline (Pro) residues of Vpr [19]. In addition, CypA also binds strongly to the seven-residue binding motif RHFP^{35}RIW centered at Pro-35 of N-terminal Vpr, which is sufficient for maintaining the strong binding of

Abbreviations: ALIX, ALG-2 interacting protein 1/X; CA, capsid; CsA, cyclosporine A; CypA, cyclophilin A; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1; L-domains, late assembly domains; NMR, nuclear magnetic resonance; NOESY, Nuclear Overhauser Effect Spectroscopy; PPIases, peptidyl–prolyl cis/trans-isomerases; Pro, proline; ROESY, Rotating-Frame Overhauser Effect Spectroscopy; SPR, surface plasmon resonance; TOCSY, Total Correlation Spectroscopy; Tsg101, tumor susceptibility gene 101; VLP, Virus like particles; Vpr, viral protein R.

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N-terminal Vpr to CypA. The functional N- and C-terminal late assembly domain (L-domain) regions of p6, namely the N-terminal PTAPP motif which interacts with tumor susceptibility gene 101 (Tsg101) [2,23–28] and the C-terminal LYPxLxxL motif which binds to Vpr [3,29] and ALG-2 interacting protein 1/X (ALIX) [30–32], contain similarly to Vpr conserved Pro residues.

With eight Pro residues, the overall Pro content of the 52-amino acid HIV-1 p6 is notably high. The PTAPP and LYPXnL late domains of p6 are involved in a cooperative manner with the nucleocapsid region of HIV-1 Gag to recruit the cellular machinery necessary for viral budding [33]. In addition to the interaction with ALIX, Pro-37 of p6 is also involved in the interaction with the viral accessory protein Vpr [9,29], which is required for the incorporation of Vpr into virus particles [3–6].

To investigate the potential role of CypA in modulating functions of HIV-1 p6 in the viral life cycle, we explored the interactions of CypA with full-length HIV-1 p6 and N- and C-terminal p6 peptides at atomic resolution by nuclear magnetic resonance (NMR) and found that CypA catalyses peptidyl–prolyl *cis*/*trans* isomerization of all Pro residues of p6. In addition, studies using Surface Plasmon Resonance (SPR) spectroscopy revealed low affinity binding of CypA to N-terminal and C-terminal regions of p6, but a higher avidity in terms of binding to full-length p6.

2. Materials and methods

2.1. Peptide synthesis

Synthesis, purification and molecular characterization of synthetic full-length $p6^{1-52}$ ($p6^{1-52}$) and p6 fragments, $p6^{1-14}$, $p6^{1-21}$, $p6^{18-29}$, $p6^{23-32}$, $p6^{32-42}$, $p6^{43-52}$ and $p6^{23-52}$ were performed as described in detail elsewhere [9,34].

2.2. Cyclosporine A and cyclophilin A

CsA was purchased from Sigma (C3662; CAS number 59865-13-3). The production and purification of recombinant human CypA have been described previously [19].

2.3. Mass spectrometry

Matrix assisted laser desorption ionization mass spectra (MALDI-MS) were recorded on a Voyager-DE PRO BioSpectrometry Workstation from Applied Biosystems. Samples were dissolved in 50% aqueous acetonitrile and α-cyano-4-hydroxycinnamic acid was used as matrix. Positive ion electrospray ionization mass spectra (ESI-MS) were recorded on a Micromass Q-Tof-2 mass spectrometer. Samples were dissolved in 70% aqueous methanol and infused into the electrospray chamber with a needle voltage of 0.9 kV at a flow rate of 40 nl/min.

2.4. NMR spectroscopy

1D and 2D NMR experiments (^{1}H TOCSY, NOESY and ROESY) were performed at 600.13 MHz on a Bruker Avance 600 MHz instrument equipped with an UltraShield Plus magnet and a triple resonance cryoprobe with gradient unit. Individual samples were dissolved in 600 μl 50 mM aqueous phosphate buffer pH 7.0, containing 10% D_2O (v/v) at concentrations between 1 and 2 mM. The 2D NMR experiments were performed at 300 K without spinning with mixing times of 110 ms for the TOCSY experiments, 250 ms for the NOESY experiments and a range of mixing times between 150 and 500 ms for the ROESY experiments, respectively. Efficient suppression of the water signal was achieved with application of excitation sculpting in the 1D ^{1}H and the 2D ^{1}H TOCSY and NOESY NMR experiments [35] and *presat* in the 2D ^{1}H ROESY experiments [36]. ^{1}H signal assignments of the NMR spectra were achieved by identification of the individual spin systems in the 2D ^{1}H TOCSY spectra, combined with observations of sequence-specific short-distance cross peaks (H_α– H_N i, i + 1) in the 2D ^{1}H NOESY spectra [37,38]. Readily recognizable spin systems were used as starting points for correlation of the individual spin systems observed in the TOCSY and NOESY spectra to individual residues in the peptide sequences. Acquisition of data, processing and spectral analysis were performed with Bruker Topspin 1.3 software. Assigned ^{1}H chemical shifts of p6 and peptides are presented in Tables A.3–A.33 of the Supplementary Data.

Table 1
Relative proportions of *cis* conformers of Pro residues of HIV-1 p6.

The relative proportions of *cis*-Pro 5, 7, 24, 30, 37 and 49 were determined for full-length p6. Relative proportions of *cis*-Pro-10 and 11 were determined for $p6^{1-14}$.

Pro residues of HIV-1 p6	Relative proportions of *cis*-Pro
Pro-5	7 ± 1%
Pro-7	7 ± 1%
Pro-10	4 ± 1%
Pro-11	3 ± 1%
Pro-24	10 ± 1%
Pro-30	10 ± 1%
Pro-37	21 ± 1%
Pro-49	5 ± 1%

2.5. Determination of time requirement for establishment of equilibrium proportions of cis and trans conformers

The initial relative proportions of *cis* and *trans* Pro-37 of $p6^{32-42}$ in H_2O–D_2O (9:1; v/v) solution were determined by integration of analogous baseline-separated signals of each conformer in the 1D ^{1}H NMR spectrum recorded immediately after dissolution of the peptide (less than 1 min). The NH signals of *cis*-Tyr-36 and *trans*-Tyr-36 were used to determine the population of each isomer of p6(32–42). Optimization of the NMR instrument (shimming, wobbling and determination of ^{1}H 90° pulse length) was performed on an identical sample of $p6^{32-42}$ in advance of the experiment. The initial 1D ^{1}H NMR spectrum was recorded with 8 scans (acquisition time 29 s). Immediately thereafter, a second 1D ^{1}H NMR spectrum was recorded with 40 scans (acquisition time 1 min 47 s), followed by a third 1D ^{1}H NMR spectrum recorded with 104 scans (acquisition time 4 min 24 s). An identical 1D ^{1}H NMR spectrum with 104 scans was finally recorded more than 24 h after sample preparation.

2.6. Influence of temperature on the relative proportions of cis and trans conformers

1D ^{1}H NMR experiments of $p6^{32-42}$ dissolved in H_2O–D_2O (9:1; v/v) were recorded at different temperatures with 10 K increments in the temperature interval 300–330 K. The initial relative proportions of *cis* and *trans* Pro-37 of $p6^{32-42}$ were determined by integration of

Table 2
Relative proportions of CypA and p6 peptides used in the NMR studies.

Peptide	Number of prolines	Peptide:CypA	Proline: CypA
$p6^{1-14}$	4	457:1	1828:1
$p6^{1-21}$	4	217:1	868:1
$p6^{23-32}$	2	481:1	962:1
		4810:1 (weak exchange peaks)	9620:1
$p6^{32-42}$	1	2167:1	2167:1
$p6^{32-42}$	1	5944:1 (strong exchange peaks)	5944:1
$p6^{23-52}$	4	80:1	320:1
$p6^{1-52}$	8	283:1	2264:1