Active-Site Residues of Cyclophilin A Are Crucial for Its Incorporation into Human Immunodeficiency Virus Type 1 Virions

TATYANA DORFMAN,¹ ANDREAS WEIMANN,¹ ALESSANDRA BORSETTI,¹
CHRISTOPHER T. WALSH,² AND HEINRICH G. GÖTTLINGER^{1,3*}

*Division of Human Retrovirology, Dana-Farber Cancer Institute,¹ Department of Biological Chemistry and Molecular Pharmacology,² and Department of Pathology,³
Harvard Medical School, Boston, Massachusetts 02115*

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Human immunodeficiency virus type 1 (HIV-1) incorporates the cellular peptidyl-prolyl *cis-trans* isomerase cyclophilin A (CyPA), the cytosolic receptor for the immunosuppressant cyclosporin A (CsA). CsA inhibits the incorporation of CyPA and reduces HIV-1 virion infectivity but is inactive against closely related primate lentiviruses that do not interact with CyPA. The incorporation of CyPA into HIV-1 virions is mediated by a specific interaction with a proline-containing, solvent-exposed loop in the capsid (CA) domain of the Gag polyprotein. CsA, which disrupts the interaction with CA, binds at the active site of CyPA. To test whether active-site residues are also involved in the interaction with HIV-1 CA, we used a panel of previously characterized active-site mutants of human CyPA. Expression vectors for epitope-tagged wild-type and mutant CyPA were transfected into COS-7 cells along with HIV-1 proviral DNA, and the virions produced were analyzed for the presence of tagged proteins. Cotransfection of the wild-type expression vector led to the incorporation of readily detectable amounts of epitope-tagged CyPA into HIV-1 virions. One CyPA mutant with a substantially decreased sensitivity to CsA was incorporated with wild-type efficiency, demonstrating that the requirements for binding to CsA and to HIV-1 CA are not identical. The remaining six CyPA mutants were incorporated with markedly reduced efficiency, providing *in vivo* evidence that HIV-1 CA interacts with the active site of CyPA.

A yeast two-hybrid screen revealed that the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein Pr55^{gag}, the precursor for the internal structural proteins of the mature virion, has the potential to interact with the host proteins cyclophilin (CyP) A and B (25). The CyPs, which were originally identified on the basis of their high affinity for the immunosuppressive drug cyclosporin A (CsA), are a family of ubiquitous peptidyl-prolyl *cis-trans* isomerases (12, 17, 31, 32, 36). CyPA is abundant in the cytosol, while CyPB has a hydrophobic leader sequence and is translocated to the endoplasmic reticulum (17, 29). *In vivo*, only the cytosolic CyPA interacts with Pr55^{gag} (14, 34), which is believed to be synthesized on free ribosomes in the cytoplasm (18). CyPA was detected in HIV-1 virions at levels comparable to those of *pol*-encoded proteins but was not found in other retroviral particles (14, 27, 34). The specific incorporation of CyPA into HIV-1 virions could be inhibited by CsA (14, 34), which also reduced the infectivity of HIV-1 virions with a similar dose dependence (34), indicating that the Pr55^{gag}-CyPA interaction is functionally relevant. Nonimmunosuppressive CsA analogs, such as SDZ NIM 811, which retain a high affinity for CyPA potentially inhibit HIV-1 replication, demonstrating that the anti-HIV-1 effect of CsA is independent of its immunosuppressive activity (2, 3, 13, 30, 34). The cyclophilin-binding compounds do not affect virus assembly or release but apparently inhibit an early step in the HIV-1 life cycle (6). Both CsA and SDZ NIM 811 are inactive against the simian immunodeficiency virus SIV_{mac},

a closely related primate immunodeficiency virus which does not interact with CyPA (30, 34).

Within Pr55^{gag}, the central capsid (CA) domain, which forms the characteristic conical core of the mature virion, is both required and sufficient for the incorporation of CyPA into virions (14, 34). The transfer of the HIV-1 CA domain to SIV_{mac} was sufficient to transfer the ability to incorporate CyPA into virions (10). Remarkably, the chimeric virus, unlike SIV_{mac}, was inhibited by the nonimmunosuppressive CsA analog SDZ NIM 811, indicating that CA, in addition to providing a CyPA binding site, is also a functional target of CyPA (10). The central role of CA in the action of CyPA is also evident from the observation that mutations in CA close to the CyPA binding site can confer resistance to or even dependence on CsA (1, 4). These mutations do not affect the ability of HIV-1 Gag to interact with CyPA but apparently reduce the requirement for the interaction for efficient virus replication (4).

CyPA interacts with a proline-containing region in CA which forms a solvent-exposed loop in the recently solved three-dimensional structure of the amino-terminal CA domain (9, 14, 16, 26). Three-dimensional structures have also been determined for unligated CyPA (22), for CyPA complexed with CsA or analogs of CsA (21, 28, 35), and for CyPA complexed with proline-containing peptides (19, 20, 38). The main structural features of CyPA are an eight-stranded antiparallel β barrel and two α helices on the top and bottom of the barrel (19, 22). Inside the barrel is a tightly packed hydrophobic core which is inaccessible to ligands; rather, the ligand binding site is located on the surface of the barrel (19, 22). The binding site for CsA is formed by 13 CyPA residues which make contacts of less than 4 Å with CsA (21, 28). These residues are contributed by strands from one side of the β barrel and by loop regions,

* Corresponding author. Mailing address: Division of Human Retrovirology, Dana-Farber Cancer Institute, Jimmy Fund Building, Rm. 824, 44 Binney St., Boston, MA 02115. Phone: (617) 632-3067. Fax: (617) 632-3113. E-mail: Heinrich_Gottlinger@DFCI.harvard.edu.

which together form a crevice on the surface of the protein. Model peptide substrates also bind in this crevice, identifying it as the active site of the enzyme (19, 20, 35, 38).

CsA specifically inhibits the virion association of CyPA, suggesting that the active site of the peptidyl-prolyl isomerase is involved in its incorporation (14, 34). To establish an experimental system in which the incorporation of transiently expressed CyPA into HIV-1 virions could be monitored, we obtained a previously described overexpression vector for epitope-tagged human CyPA (7). The CyPA coding sequence in this vector is under the control of the highly active SR α promoter (33) and is fused to a sequence which adds an influenza virus hemagglutinin (HA) epitope tag to the carboxy terminus of CyPA (7). The vector also contains a simian virus 40 T-antigen-dependent replication origin. To produce HIV-1 virions, we used the proviral construct HXB/Vpr⁺/PR⁻, a version of the infectious HXBH10 molecular clone in which the reading frame for the virion-associated regulatory viral protein Vpr was restored (8). A point mutation in the protease gene of HXB/Vpr⁺/PR⁻ prevents cleavage of Pr55^{gag}, which facilitates the detection of nonstructural virion-associated proteins (8).

HXB/Vpr⁺/PR⁻ proviral DNA was transfected into COS-7 cells along with the expression vector for HA-tagged CyPA (CyPA-HA) or with a variant with a frameshift mutation in the CyPA coding sequence. The frameshift mutation was generated by inserting 1 bp at the unique *Tth*111I site in the human CyPA cDNA with the Klenow fragment of DNA polymerase I. The cells were metabolically labeled with [³⁵S]methionine from 48 to 60 h posttransfection, lysed in radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), and immunoprecipitated with anti-HA monoclonal antibody 16B12 (Babco, Richmond, Calif.). As shown in Fig. 1A, a protein with the expected electrophoretic mobility of HA-tagged CyPA was detected in cells transfected with the CyPA-HA expression vector (lane 1) but not in cells transfected with the control vector (lane 2).

To determine whether HA-tagged CyPA was incorporated into virions, particulate material released during the labeling period was partially purified by ultracentrifugation through 20% sucrose. The pelleted material was lysed in RIPA buffer and analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. Cotransfection of the CyPA-HA expression vector along with the HXB/Vpr⁺/PR⁻ proviral construct resulted in the appearance of a novel protein in HIV-1 virions which comigrated exactly with CyPA-HA immunoprecipitated from cell lysates (Fig. 1B, lane 1) and which could be immunoprecipitated from virion preparations with the anti-HA monoclonal antibody (data not shown). Because of its slower electrophoretic mobility, CyPA-HA could readily be distinguished from endogenous CyPA after direct analysis of virion preparations by SDS-PAGE (Fig. 1B). Under the conditions used, the band for CyPA-HA was more prominent than that for endogenous CyPA, which may in part reflect more efficient metabolic labeling of the transiently expressed form.

To determine whether the ligand binding site of CyPA is critical for its incorporation into HIV-1 virions, we made use of a panel of seven active-site mutants of human CyPA which were previously assayed for their *cis-trans* peptidyl-prolyl isomerase activity as well as for their ability to bind CsA (24, 39). The locations of the mutated residues in the three-dimensional structure of CyPA are indicated in Fig. 2. The R55A, F60A, and H126Q mutants retain less than 1% of the wild-type catalytic efficiency in a tetrapeptide assay, while the H54Q, Q111A, and F113A mutants retain between 3 and 15%, and

FIG. 1. Incorporation of epitope-tagged CyPA into HIV-1 virions. COS-7 cells were cotransfected with the protease-defective proviral construct HXB/Vpr⁺/PR⁻ and a HA-tagged CyPA expression vector (lanes 1) or a control vector with a frameshift mutation in the CyPA coding sequence (lanes 2). (A) The cells were metabolically labeled with [³⁵S]methionine from 48 to 60 h posttransfection and lysed in RIPA buffer, and HA-tagged protein was immunoprecipitated from the cell lysates. (B) Virions released during the labeling period were sedimented through 20% sucrose and disrupted in RIPA buffer, and virion-associated proteins were directly analyzed by SDS-PAGE. The positions of migration of molecular mass markers (in kilodaltons) are indicated on the right. The positions of specific proteins are indicated on the left.

the rotamase activity of the W121F mutant is reduced only by about twofold (24, 39). CsA binding is most affected by the F60A, F113A, and H126Q mutations (39). The W121F mutation is of particular interest because it causes a 75-fold decrease in sensitivity to CsA, despite its relatively moderate effect on catalytic efficiency, separating peptidyl-prolyl isomerase activity from CsA binding (24).

Fragments carrying these mutations were transferred from bacterial expression vectors into the mammalian CyPA-HA expression vector by using unique *Tth*111I and *Eco*47III sites within the human CyPA coding sequence. The wild-type and mutant CyPA-HA expression vectors were transfected into COS-7 cells, followed by metabolic labeling with [³⁵S]methionine for 12 h. Immunoprecipitation from the cell lysates with the anti-HA antibody revealed that the H54Q mutant yielded about 10-fold lower steady-state levels of HA-tagged protein than the wild-type CyPA-HA expression vector; the other mutants yielded levels which were more comparable to that obtained with the wild-type vector (data not shown). To test for incorporation of the CyPA mutants into HIV-1 virions, various amounts of the CyPA-HA expression vectors were transfected into COS-7 cells in combination with HXB/Vpr⁺/PR⁻ proviral

FIG. 2. Locations of mutated residues in the three-dimensional structure of CyPA. The ribbon model of CyPA and the ball-and-stick models of residues His 54, Arg 55, Phe 60, Gln 111, Phe 113, Trp 121, and His 126 were drawn by using the program MOLSCRIPT (23).

DNA. As a control, the wild-type CyPA-HA expression vector was cotransfected with HXBH10-*gag*[−], a HIV-1 proviral construct which is unable to produce viral particles because it lacks an open *gag* reading frame (11). The appearance of HA-tagged CyPA in the particulate fraction was dependent on the presence of a functional *gag* gene, confirming its association with viral particles (Fig. 3A and B, lanes 1). By adjusting the amounts of transfected vector DNA, intracellular steady-state levels could be achieved for each of the CyPA mutants which were comparable to or even higher than those obtained with the wild-type CyPA-HA expression vector (Fig. 3C). Nevertheless, virions produced in the presence of the H54Q, R55A, F60A, Q111A, F113A, and H126Q mutants contained substantially lower amounts of tagged HA-CyPA than virions produced in the presence of the wild-type construct (Fig. 3A and B). Taking into account relative intracellular steady-state levels, these mutants were incorporated with at least 10-fold lower efficiency than wild-type CyPA-HA. In contrast, the W121F mutant was incorporated with wild-type efficiency (Fig. 3A and B, lanes 6).

Our analysis shows that six of the seven mutated residues in the active-site groove of CyPA are critical for its incorporation into HIV-1 virions. Our results are consistent with the recently solved crystal structures of CyPA bound to the N-terminal domain of HIV-1 CA (15) and to a 25-mer fragment of HIV-1 CA (37). The structure of CyPA bound to the N-terminal CA domain reveals that the CA-CyPA interface is formed exclusively by an exposed proline-containing CA loop that binds in the active site of the isomerase (15). Pro90 of CA, which is crucial for the virion incorporation of CyPA (14), occupies the same hydrophobic pocket in CyPA as prolines in model peptide substrates or the MeVal11 side chain of CsA (15, 37). The hydrophobic pocket is primarily formed by CyPA residues F60,

M61, F113, L122, and H126 (37). We targeted three of these residues and found that all three are critical for the virion association of CyPA. We also targeted four CyPA residues which are involved in hydrogen-bonding interactions with the exposed CA loop, either directly or through a bridging water molecule (15). Of these, H54, R55, and H126 are crucial for the incorporation of CyPA, while W121 is not. Consistent with our results, Braaten et al. recently showed that the H54Q, R55A, F60A, F113A, and H126Q mutations drastically reduce CyPA binding to Pr55^{gag} in vitro and also observed that these mutations inhibit CyPA incorporation into HIV-1 virions (5).

Interestingly, W121 is a major determinant in CsA recognition (24). Since the W121F mutant exhibits resistance to CsA but retains enzymatic activity and is efficiently incorporated into HIV-1 virions, this mutant may be useful for assessing whether the specific anti-HIV-1 activity of CsA is a direct consequence of its inhibitory effect on the HIV-1 Gag-CyPA interaction.

FIG. 3. Effects of active-site mutations on CyPA incorporation. COS-7 cells were transfected with HXBH10-*gag*[−] (lanes 1 and 8) or HXB/Vpr⁺/PR[−] (lanes 2 to 7 and 9 to 14) along with the indicated CyPA-HA expression vectors. Transfected cells were metabolically labeled with [³⁵S]methionine for 12 h, and viral particles released during the labeling interval were analyzed directly by SDS-PAGE (A) and in parallel by immunoprecipitation with anti-HA antibody 16B12 (B). CyPA-HA was also immunoprecipitated from the lysates of transfected cells to compare intracellular steady-state levels (C). The amounts of transfected vector DNA were adjusted to achieve expression levels for each CyPA mutant which were at least as high as those obtained with the wild-type vector. The amounts of vector DNA used (in micrograms) were 30 (lane 1), 7 (lane 2), 15 (lane 3), 30 (lane 4), 20 (lane 5), 15 (lane 6), 20 (lane 7), 4 (lanes 8 and 9), 20 (lane 10), 40 (lane 11), and 15 (lanes 12 to 14).

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