Cyclophilin Binding to the Human Immunodeficiency Virus Type 1 Gag Polyprotein Is Mimicked by an Anti-Cyclosporine Antibody

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Human immunodeficiency virus type 1 is unique among retroviruses in that infectivity requires specific incorporation into virions of the cellular protein cyclophilin A through interactions with the Gag polyprotein. Here we show that monoclonal antibody B11 1.4, which recognizes a cyclophilin-binding epitope on cyclosporine, detects denatured or native human immunodeficiency virus type 1 capsid. B11 1.4 does not recognize the capsids of other retroviruses, and binding is inhibited by cyclosporine or by cyclophilin A.

Previously, we discovered an interaction between the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein and a family of ubiquitous cellular proteins known as the cyclophilins (11). Cyclophilins bind the immunosuppressive drug cyclosporine (8) and as a complex with the drug inhibit the activity of calcineurin, a phosphatase necessary for T-cell activation (14). The native function of cyclophilins is perhaps related to their ability to catalyze the isomerization of peptidyl-prolyl bonds, a rate-limiting step in protein folding (5). Cyclophilins also protect cells from heat shock (16) and function as chaperones (3).

Unlike cyclosporine, HIV-1 Gag does not promote the assembly of an immunosuppressive complex with cyclophilin and calcineurin (11), but the proper folding or cellular trafficking of HIV-1 Gag may require the cyclophilin interaction. Cyclophilin A, the major cytoplasmic cyclophilin, binds to the HIV-1 Gag polyprotein in vitro and is specifically incorporated into HIV-1 virions (6, 11). Gag mutants or cyclosporine-related compounds which disrupt the Gag-cyclophilin interaction in vitro disrupt incorporation of cyclophilin A into virions and block HIV-1 infectivity in tissue culture (6, 17). Thus, cyclophilin A appears to be required for HIV-1 virion infectivity.

Since HIV-1 Gag and cyclosporine compete in cyclophilin binding reactions (11), we hypothesized that they display a common epitope recognized by cyclophilin. This element need not have peptide sequences in common but would share a three-dimensional structure. To identify this putative epitope we examined a collection of 22 monoclonal anticyclosporine antibodies which had previously been characterized with respect to the epitope recognized on cyclosporine (1). A hybridoma supernatant for each monoclonal antibody was tested for reactivity in Western blots (immunoblots) against bacterially expressed HIV-1 Gag polyprotein as previously described (12). As a positive control the blot was probed with a monoclonal anticapsid (p24) antibody (Dupont) (Fig. 1, lane 1) which revealed the position of migration of Pr55^{gag} as well as those of previously characterized carboxy-terminal degradation products (12). Of the anticyclosporine antibodies tested, only one, the immunoglobulin M(κ) antibody B11 1.4, demonstrated significant reactivity with Pr55^{gag} (Fig. 1, lane 9). Interestingly, B11 1.4 was previously shown to recognize a cyclosporine

epitope made up of the 9,10-*N*-methyl-leucyl-*N*-methyl-leucine dipeptide, sequences known to make direct contact with cyclophilin A (1).

To determine if B11 1.4 reactivity is specific for Gag polyproteins capable of binding to cyclophilin, we took advantage of previous work examining the abilities of different viral Gag polyproteins to bind to the cyclophilins. First we demonstrated that B11 1.4 did not react with bacterially expressed Gag polyprotein of Mason-Pfizer monkey virus (data not shown). This Gag polyprotein does not bind to any of the cyclophilins (11). Next we demonstrated that the antibody did not react with the Gag polyprotein of a simian immunodeficiency virus (SIV) strain, SIV_{MAC239} (Fig. 2, lane 3), which was expressed in bacteria as previously described (6). Coomassie gels (data not shown) and Western blots with an antibody that recognizes the capsids of HIV-1, HIV-2, and SIV_{MAC239} (6) demonstrated that comparable amounts of Gag were loaded in each lane (Fig. 2, lane 7). While the Gag polyproteins of all primate lentiviruses bind to cyclophilin B, only the HIV-1 Gag polyprotein binds to cyclophilin A. Cyclophilin B has a signal sequence which targets it to the endoplasmic reticulum (7), where it seems unlikely to contact the predominantly cytoplasmic Gag polyprotein. In addition, cyclosporine has no effect on the replication of retroviruses such as SIV_{MAC239} (13, 17), suggesting that the interaction of Gag with cyclophilin B is of no importance to viral replication. Thus, among retroviral Gag polyproteins, B11 1.4 is specific for HIV-1 Gag, the only one capable of binding cyclophilin A.

Previous analysis of the gag primary sequence requirements for cyclophilin A binding demonstrated that capsid coding sequences retain information sufficient for binding (11). Further evidence that B11 1.4 might recognize a Gag epitope relevant for cyclophilin binding was provided by the observation that the antibody reacted strongly with p24 (Fig. 2, lane 1), which was expressed in bacteria as previously described (11).

To date we have identified a single gag point mutation, a change from proline to alanine at position 222 (P222A), which disrupts the interaction with cyclophilin A (6). B11 1.4 recognizes Gag protein containing the P222A mutation (Fig. 2, lane 4), suggesting that there may be additional points of contact between Gag and cyclophilin A.

B11 1.4 detected not only bacterially expressed HIV-1 Gag proteins but also HIV-1 virion-associated p24 (Fig. 3, lane 1), which was purified from the supernatant of transiently transfected 293 cells as previously described (6). Similar levels of

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FIG. 1. Western blot screen of monoclonal anticyclosporine antibodies for binding to the HIV-1 Gag polyprotein. Protein from a lysate of bacteria expressing Pr55^{gag} was size separated on a sodium dodecyl sulfate-polyacrylamide gel and blotted onto nitrocellulose as previously described (12). The nitrocellulose was cut into strips, each of which was probed with a different primary antibody. Lane 1 was probed with a monoclonal anti-HIV-1 capsid antibody. Lanes 2 to 23 were probed with supernatants from anticyclosporine hybridomas. Lane 9 was probed with monoclonal B11 1.4. Lane 24 was probed with no primary antibody. The position of migration of Pr55^{gag} is indicated on the left.

binding were observed with Pr55^{gag} purified from protease-defective virions (6), with or without the P222A mutation (Fig. 3, lanes 3 and 4). The reactivity was specific for HIV-1 capsid, since B11 1.4 did not recognize virion-associated capsid from HIV-2_{ROD} or Moloney murine leukemia virus (Fig. 3, lanes 2 and 5).

Attempts to immunoprecipitate virion-associated p24 with B11 1.4 were unsuccessful. To demonstrate that the B11 1.4 monoclonal antibody reacts with nondenatured p24, we set up an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates (Corning 25855) were coated with recombinant p24 produced in a baculovirus expression system (American Biotechnologies, Inc., Cambridge, Mass.) at 1 µg/ml in 0.1 N NaHCO₃, pH 9.3, for 2.5 h at 37°C. The plates were then blocked with 2% fetal calf serum in phosphate-buffered saline

(PBS) with 0.1% Tween 20 for 1 h at 37°C. B11 1.4 ascites fluid was preincubated with cyclosporine (Sandoz Research Institute) at various concentrations (Fig. 4A) for 1 h at 37°C and then incubated with the p24-coated plates for 90 min at 37°C. Bound B11 1.4 was then detected in a peroxidase reaction as previously described (1). The specificity of B11 1.4 binding to native p24 was demonstrated by the linear inhibition of binding by cyclosporine (Fig. 4A).

Lastly, we attempted to block antibody binding by preincubating the p24-coated plates with purified human cyclophilin A (a generous gift from Robert E. Handschumacher, Yale University) at various concentrations in PBS for 1 h at 37°C. We observed a linear inhibition of antibody binding by cyclophilin A (Fig. 4B), indicating either that cyclophilin competed with the antibody for its binding site on p24 or that cyclophilin binding to p24 sterically blocked the antibody binding site.

FIG. 2. Binding of B11 1.4 to bacterially expressed Gag proteins. Lanes 1 to 4 are from a Western blot probed with B11 1.4. As a control for protein expression, the same samples were probed with a monoclonal antibody which recognizes the capsids (CA) of HIV-1 and SIV_{MAC239} (6) (lanes 5 to 8). Individual lanes contain lysates from bacteria expressing HIV-1_{HXB2} p24 (lanes 1 and 5), HIV-1_{HXB2} Pr55^{gag} (lanes 2 and 6), SIV_{MAC239} Pr55^{gag} (lanes 3 and 7), and HIV-1 Pr55^{gag} with the P222A mutation (lanes 4 and 8). The positions of migration of molecular size markers are indicated.

FIG. 3. Binding of B11 1.4 to virion-associated capsid. Virion proteins were purified from the supernatant of 293 cells transfected with HIV-1 pNL4-3 (lanes 1 and 6), HIV2_{ROD} (lanes 2 and 7), pSRα55 (lanes 3 and 8), or pSRα55-P222A (lanes 4 and 9) as previously described (6). Moloney murine leukemia virus proteins (lanes 5 and 10) were obtained from NIH 3T3 cells transfected with pNCA (2). Viral proteins were probed with B11 1.4 (lanes 1 to 5) or with two anticapsid (anti-CA) antibodies (lanes 6 to 10): one recognizes the capsids of both HIV-1 and HIV-2 (6), and the other recognizes the capsid of Moloney murine leukemia virus (N.C.I. 79S 804). The positions of migration of molecular size markers are indicated.

FIG. 4. Inhibition of binding of B11 1.4 to nondenatured p24 by cyclosporine (CsA) or by cyclophilin A (CyP A). Binding of B11 1.4 to p24 expressed in baculovirus was measured in an ELISA format after the antibody was preincubated with cyclosporine at the indicated concentrations (A) or after the p24-coated wells were preincubated with purified cyclophilin A at the concentrations indicated (B). Data are presented as percent inhibition of binding in the absence of an inhibitor.

We have demonstrated that a monoclonal antibody raised against cyclosporine cross-reacts with the Gag polypeptide of HIV-1. Identification of such an antibody is not surprising given the fact that cyclosporine and HIV-1 Gag compete with each other for binding to cyclophilin A (11). Our data suggest that B11 1.4 recognizes an epitope important for cyclophilin A binding, and this antibody may help identify the structures in Gag required for this binding reaction.

The three-dimensional structure of cyclosporine bound to cyclophilin A has been determined by several groups using both X-ray and nuclear magnetic resonance data (for examples, see references 10 and 15). B11 1.4 recognizes cyclosporine positions 9 and 10, which are both residues that have been proposed to form direct contacts with cyclophilin upon binding (4, 18). Interestingly, significant structural alterations occur at

residues 9 and 10 upon binding to cyclophilin (9, 19): when the drug is free in solution, a *cis* peptide bond exists between the two *N*-methyl-leucines at positions 9 and 10; upon binding to cyclophilin this bond converts to the *trans* conformation. Perhaps similar transformations occur in the structure of HIV-1 Gag upon binding of the protein to cyclophilin A.

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