Interaction of the Capsid Protein p24 (HIV-1) with Sequence-Derived Peptides: Influence on p24 Dimerization

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Protein–protein interactions of the p24 (HIV-1) capsid protein play an essential role in the production of infectious virus particles. To map the putative p24 dimerization site, a set of overlapping peptides spanning the p24 sequence was prepared using spot synthesis on a cellulose membrane and probed with recombinant p24 (rp24). Three sequence regions interacting with rp24 were identified. Peptides from each region were synthesized, but only one peptide was effectively able to inhibit rp24 dimerization in solution. Amino acids that were exposed in the corresponding p24 region were mutated in rp24, resulting in a significant decrease of rp24 dimerization. Thus, participation of this region in virus capsid assembly can be assumed.

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N-terminal sequence (here MNSAM... instead of PI...) seems to have little influence on the monomer/dimer dissociation constant, suggesting that the proline at sequence position 1 of wild-type p24 that forms a salt bridge with D-51 in the mature p24 structure (9) plays no significant role in its dimerization behavior.

To locate linear sequence parts of the discontinuous dimerization site involved in the interaction between rp24 monomers we used spot synthesis to generate a peptide scan of overlapping p24-derived peptides (13mers with a 10-amino-acid overlap). The cellulose-bound peptides were screened for p24 binding by incubation with recombinant p24 (rp24). Bound protein was subsequently transferred by electroblotting onto a PVDF membrane. This facilitated the detection of bound rp24 even in the case of comparatively low affinities by conventional immunoblotting techniques using a murine anti-p24 monoclonal antibody and avoided interference of the antibody with membrane-bound peptides. A blot of the probed peptide scan, together with the corresponding densitometric analysis and sequences of the relevant peptides, is presented in Fig. 1. Three clearly defined sequence regions showed significant interactions with rp24: a region comprising the peptides 54–57 (p24 residues 157 to 180) with the strongest interaction, peptides 47–49 (p24 residues 136 to 154) with somewhat weaker binding, and peptides 42 and 43 (p24 residues 121 to 136) with the weakest signal.

To decide whether the sequence regions that bind rp24 are involved in p24 dimerization, represent additional potential contact sites involved during capsid assembly, or simply mediate nonspecific interactions, free peptides selected from those regions were used to monitor their influence on p24 dimerization in solution. In addition, peptides derived from putative p24 interaction sites described in the literature were included in the analysis. The following peptides were chosen: peptide 1 (IPVGEIYKRW; sequence position 124–133) derived from region 1 of the peptide scan; peptide 2 (VRMYSPT); sequence position 142–149) from region 2; and peptide 3 (PFRDYVDRFYKTL; sequence position 160–172) from region 3. The length of these peptides was based on the binding profile of rp24 to the various overlapping sequences, but neighboring hydrophobic residues, which would reduce the peptide solubility, were omitted. In addition, peptide 4 (PAATLEEMMTA; sequence position 207–217) from the p24 C-terminal domain, which was reported to inhibit HIV virus spread in cell cultures (14), and three peptides from the putative dimerization sites derived from X-ray structures were included. These three additional peptides are peptide 5 (DIAGTTSTLQEQ; position 103–114 in p24 N-terminal domain) (12); peptide 6 (AFSPEVIPMFSAL; position 31–43) (10); and peptide 7 (QEVKNMWTETLL; position 179–190) (11).

Analytical ultracentrifugation was used to investigate the influence of peptides on the monomer/dimer equilibrium of rp24 in solution. An intermediate concentration of the protein was chosen for all experiments (about 8 μM). The apparent average molecular mass of rp24 in the absence of peptides was calculated to be between 40 and 42 kDa at this concentration. Peptides were added up to a 35-fold molar excess over the rp24 concentration before ultracentrifugation. From the sedimentation equilibrium measurements the weight average molecular mass ($M_w$) of rp24 was calculated. The results are shown in Fig. 2. Peptides 2 to 7 had no significant effect on the $M_w$ values of rp24. Since preliminary experiments with peptide 1 indicated a clear influence on the sedimentation behavior of rp24, more detailed analyses were performed testing various concentrations of this peptide. From the concentration-dependent influence of peptide 1 on the monomer/dimer ratio of rp24 and taking into account the equilibrium constant of this process, a dissociation constant of $3.7 \pm 0.1 \times 10^{-5}$ M was calculated for the interaction of peptide 1 with rp24.

Suppression of p24 dimerization by a sequence-derived peptide does not necessarily mean that this sequence region is involved in the dimerization site. Therefore, mutations were introduced into rp24 to ascertain the importance of such a site. From an inspection of the
region corresponding to peptide 1 in the p24 N-terminal domain structure, there are mainly two residues that are candidates for being involved in p24–p24 interaction: E128 and R132. In our study E128 was mutated to alanine (rp24/E128A) or arginine (rp24/E128R), and R132 was mutated to glutamate (rp24/R132E).

In the ultracentrifugation experiments the first two mutants show a significantly lower tendency for dimerization, with the dimerization constant differing by a factor of about 4–5 for the E128A mutant (Fig. 3) and of about 2 for the E128R mutant in comparison with the constant for wild-type rp24 (data not shown). The third mutation, R132E with R132, had no influence on the rp24 dimerization behavior (Fig. 3). There were no differences between the CD spectra of the wild-type rp24 and refolded mutant proteins (data not shown). Their stability toward GuHCl denaturation as measured by the shift in fluorescence emission spectra was slightly lower than that of the wild-type rp24 (data not shown).

Analyses of overlapping peptides spanning the entire p24 sequence revealed three different p24 regions binding to the recombinant p24 protein. All three regions contain a number of highly conserved positions when sequences from different HIV-1, HIV-2, and SIV isolates are compared. For instance, region 3 comprises a sequence with conserved positions accumulated in all retroviral gag genes, the so-called "major homology region" (MHR, positions 153 to 173 in the p24 sequence (15)).

The region represented by peptide 1 (sequence position 124 to 133) is part of helix VII in the N-terminal domain of the p24 structure (9). In addition, it has been shown by Niedrig et al. (14) that a peptide from this region is able to inhibit the virus spreading in cell culture, albeit less effectively than the peptide from the C-terminal part of p24, which was also included in the experiments described here (peptide 4). Results of an amino acid deletion at positions 132 to 136, overlapping with the last two residues of peptide 1, revealed a reduced virus replication, but the mutant particles are able to assemble and can release viral particles. It was shown that the morphology of the particle is changed (16).

In recent reports on partial p24 structures solved by X-ray analysis (N-terminal domain, (10, 12); C-terminal domain, (11)) there are four different proposed dimerization sites (Fig. 4). For the complex between anti-p24 Fab fragment and p24 (12) the sequence region corresponding to peptide 1 does not fit the interface region between two p24 molecules in the crystal structure. Two interface regions (p24 positions 18 to 29/30 to 45 and positions 132 to 143) are depicted by X-ray structure analysis of the cyclophilin A/N-terminal p24 fragment complex (10). The region corresponding to p24 sequence positions 132 to 143 lies exactly between our peptide 1 and 2 sequences and overlaps at both ends by two amino acids each (p24 positions 132/133 and 142/143). This means that at least the sequence region of peptide 1 with its inhibitory effect on rp24 dimerization lies in the vicinity of a crystallographically detected p24–p24 interface. All other peptides derived from putative dimerization sites (peptides 5, 6, and 7) had no influence on the p24 dimerization behavior. Certainly not all the interface regions derived from the crystal structures need be involved in the dimerization process in solution or the capsid assembly in vivo; they may simply be a result of crystal packing. Only for the interface region of the C-terminus was it shown that

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**FIG. 2.** Influence of peptides on rp24 dimer formation. The apparent molecular weight of rp24 in the presence of varying concentrations of different peptides was measured by sedimentation equilibrium centrifugation. The indicated dimer and monomer limits were derived from the theoretical molecular weights: (■) Peptide 1 (IPVGEIYKRW); (☐) peptide 2 (VMYPSPTS); (△) peptide 3 (PFDRYVDRFYKTL); (○) peptide 4 (PAATL-EEMMTA); (☒) peptide 5 (DIAGTTSTLQEQ); (+) peptide 6 (AFSVPEVIFMFSAL); (++) peptide 7 (QEVKMWMETETLL).

**FIG. 3.** Concentration dependence of dimer formation for wild-type rp24 and mutants as measured by analytical ultracentrifugation. (■) Wild-type rp24; (□) E128A mutant; (☒) R132E mutant. The bars indicate the variation of values as calculated from different points of measurement within one experiment.
mutations introduced within the putative interaction region abolished the dimerization of the complete p24 at concentrations up to 0.25 mg/ml (11). Our analytical ultracentrifugation data with a M184A mutant clearly showed the occurrence of dimers at concentrations higher than 1 mg/ml. That the peptide derived from this region (peptide 7) does not influence the dimerization behavior of p24 may be due to a low affinity of the peptide for p24. This may also hold true for peptides derived from other regions except that of peptide 1.

Inspection of the sequence region corresponding to peptide 1 in the p24 structure led to the identification of two potential key residues of interaction: both E128 (position 5 in peptide 1) and R132 (position 9 in peptide 1) are solvent exposed. But only the E128A and E128R mutants of rp24 show diminished dimerization tendency without any detectable influence on the overall p24 folding as judged by CD. Therefore, this residue seems to be of special importance in p24 intermolecular contacts in addition to the role of the C-terminal domain described in the literature (11).

Derivatives of peptide 1 are currently being tested in cell cultures to investigate their inhibitory effect on HIV virus spreading. Furthermore, a p24 double-mutant containing substitutions in the sequence region corresponding to peptide 1 and peptide 7 is under construction to suppress dimerization even at high protein concentrations necessary for crystallization experiments or NMR studies with the complete protein.

Mutations were introduced using Chameleon Double-Stranded Site-Directed Mutagenesis Kit (Stratagene, La Jolla) according to the manufacturer’s protocol except that the temperature was kept at 28°C. All details about the vector construction, as well as the expression and purification procedure, were described by Hausdorf et al. (13).

Peptide scans were automatically synthesized (Abimed, Lengenfeld) as previously described (17). The membranes were blocked by incubation with a solution
of 3% dry milk powder in Tris-buffered saline, pH 7.5, containing 1% Tween 20 (TTBS) for 2 h at room temperature, washed three times with TTBS for 5 min each, and incubated for 3 h at room temperature with 75 μg/ml rp24 in 50 mM citrate buffer, pH 6.0, containing 0.15 M NaCl. The membranes were finally washed with TTBS for 4 min. The bound protein was electroblotted to a polyvinylidene difluoride (PVDF) membrane at 0.8 mA/cm² for 30 min. The bound protein was electroblotted to a polyvinylidene difluoride (PVDF) membrane at 0.8 mA/cm² for 30 min. The PVDF membranes were blocked by incubation with a solution of 3% dry milk powder in TTBS for 30 min and subsequently washed three times with TTBS for 5 min each. Bound rp24 was detected using the murine monoclonal anti-p24 antibody CB4-1 (2 μg/ml) and a sheep anti-mouse IgG horseradish peroxidase conjugate (Amersham, Freiburg) (dilution 1:1000). 3,3′-Diaminobenzidine tetrahydrochloride (0.8 mg/ml) (Sigma, Deisenhofen) in 100 mM Tris–HCl, pH 7.5, with 0.2% NiCl₂ and 0.03% H₂O₂ was used as the peroxidase substrate.

Standard protocols were used for the covalent coupling of rp24 (11 mg) to BrCN–Sepharose (Pharmacia, Freiburg) (0.7 g) in 0.5 M sodium bicarbonate buffer containing 0.5 M NaCl at pH 8.0. Zonal elution profiles for rp24 at a concentration of about 8 mg/ml were obtained by injecting rp24 at a flow rate of 0.25 ml/min. The determination of the dissociation constant for the dimeric complex was performed as described by Rosé et al. (8).

Sedimentation equilibrium measurements were carried out using a Beckman XL-A ultracentrifuge with UV absorbance optics and six-channel cells. About 70 μl of rp24 at a concentration of about 8 μM in 50 mM citrate buffer, pH 6.0, containing 0.15 M NaCl was aliquoted in the absence or in the presence of different amounts of the appropriate peptide into the different compartments of the six-channel cells and centrifuged at 30,000 rpm to accelerate the concentration distribution followed by an equilibrium speed of 24,000 rpm for 22–24 h. All measurements were carried out at 10°C. The radial concentration distributions at the sedimentation equilibrium were recorded by measuring the absorption against buffer at 280, 285, and 290 nm. Using the program “Polypro” (13), the weight average molecular mass M_w of rp24 was calculated.

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