Conformational changes between human immunodeficiency virus type 1 nucleocapsid protein NCp7 and its precursor NCp15 as detected by anti-NCp7 monoclonal antibodies

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The nucleocapsid protein NCp15 of human immunodeficiency virus type 1 (HIV-1) is a small basic protein with two zinc fingers. It is required for virion morphogenesis and synthesis of proviral DNA. As the first step in our study of the structural domains involved in the various functions of this protein, 18 monoclonal antibodies (MAbs) were isolated. The epitopes of NCp7 recognized by the MAbs were mapped using synthetic peptides representing overlapping sequences and truncated forms of NCp7. These anti-NCp7 MAbs were investigated by ELISA and real-time biospecific interaction analysis (BIAcore). Five classes of anti-NCp7 MAbs were characterized. Three classes (14 MAbs) were directed against continuous epitopes, one in the N-terminal part, another next to the second zinc finger and the third in the C-terminal part of the protein. Two other classes comprised four MAbs reacting only with the entire NCp7 and not with any of the small overlapping peptides used, suggesting that these MAbs were directed against conformational epitopes. The anti-NCp7 MAbs directed against linear epitopes were able to react efficiently with both NCp7 and NCp15, the NCp7 precursor, whereas the anti-NCp7 MAbs directed against conformational epitopes did not react with NCp15. Interestingly, most of the anti-NCp7 MAbs directed against conformational epitopes were capable of inhibiting the tight interaction between NCp7 and the HIV-1 replication primer tRNAlys3. In contrast, most of the MAbs directed against linear epitopes did not inhibit this interaction.

Introduction

The genomic RNA in the virion core of retroviruses is closely associated with nucleocapsid (NC) protein molecules, forming the nucleocapsid (Darlix et al., 1990; Fleissner & Tress, 1973; Méric et al., 1984). The NC protein (NCp7) of human immunodeficiency virus type 1 (HIV-1), which is part of the C-terminal domain of the polyprotein Pr55gag (Peterlin & Luciw, 1988; Di Marzo Veroneze et al., 1987, 1988), is a small basic peptide of 72 amino acids containing two copies of a CX3CX2HXC4 zinc finger-like motif (Wain-Hobson et al., 1985) flanked by several basic residues. This zinc finger motif is also found in the NC protein of other retroviruses (Green & Berg, 1989).

Each finger has a high affinity for one equivalent of zinc (Cornille et al., 1990; Fitzgerald & Coleman, 1991; South et al., 1991). 1H nuclear magnetic resonance (NMR) spectroscopy and molecular modelling have shown that the two zinc fingers are close together, the N- and C-terminal sequences behaving as flexible independent domains (Morellet et al., 1992, 1994; Ominchinski et al., 1991).

NC protein promotes viral RNA dimerization in vitro (Darlix et al., 1990; De Rocquigny et al., 1992). This process is probably associated with genomic RNA packaging in vivo (Housset et al., 1993; Gorenflick et al., 1990; Aldovini & Young, 1990), since dimerization and packaging require the same trans-acting factor, the protein, and ets-element, the packaging sequence (Darlix et al., 1990). NCp7 also activates the annealing of replication primer tRNAlys3 to the genomic RNA at the initiation site for reverse transcription (primer binding site) in vitro (Barat et al., 1989) and is thus required for
the initiation of cDNA synthesis (Barat et al., 1991, 1993). Lastly, NCp7 forms stable complexes with proviral DNA sequences and protects these from nuclease digestion (Lapadat-Talposky et al., 1993).

In order to map the functional domains of HIV-1 NCp7, MAbs have been raised in mice and rats. Recently, we reported that an anti-NCp7 MAb could inhibit the tight binding of NCp7 to tRNA<sub>lys</sub> as well as viral RNA dimerization and strong-stop cDNA synthesis induced by NCp7 (Tanchou et al., 1994). In the present work, we describe the extensive characterization of 18 anti-NCp7 MAbs. These MAbs have been mapped and five classes distinguished by analysing their reactivity against various synthetic peptides representing different domains of the protein. Their ability to react with NCp15, the immediate precursor of NCp7, as well as their inhibitory activity on the tight binding of NCp7 to tRNA<sub>lys</sub> have also been determined. We found that none of the anti-NCp7 MAbs directed against conformational epitopes reacted with NCp15 and that all but one were capable of inhibiting the NCp7 tight binding to tRNA<sub>lys</sub>. In contrast, all the MAbs directed against linear epitopes of NCp7 reacted with NCp15 and most of them did not inhibit the binding of NCp7 to tRNA<sub>lys</sub>.

**Methods**

**Synthetic peptides and recombinant proteins.** NCp7 and derived peptides (Fig. 1) were synthesized by a solid-phase method as previously described (De Rocquigny et al., 1991). Small overlapping peptides were provided by the Agence Nationale de Recherches sur le SIDA (ANRS; Paris, France). Prior to use, lyophilized NCp7 and NCp7 peptides were resuspended at 1 mg/ml in sterile double-distilled water and blocked with PBS containing 1% skimmed milk and 0.1% Tween 20 for 30 min at 37°C. Hybridoma cell culture supernatants (diluted 1:10) were pre-incubated for 30 min at 37 °C. Hybridoma cell culture supernatants (diluted 1:10) were pre-incubated for 2 h at 37 °C. In competition experiments, culture supernatants (diluted 1:10) were pre-incubated with 5 μg of the corresponding peptides overnight at 4 °C in 50 μl. Affinity purified biotinylated anti-mouse IgG (Dako) or anti-rat IgG (Dako) were used as secondary antibodies.

**ELISA.** Wells of Dynatech plates were coated overnight at 4 °C with 5 ng/ml of either NC protein or NC peptides in 0.1 M-Na<sub>2</sub>CO<sub>3</sub> pH 10 and blocked with PBS containing 1% skimmed milk and 0.1% Tween 20 for 30 min at 37 °C. Hybridoma cell culture supernatants (diluted 1:2) were added to each well and incubated for 2 h at 37 °C. In competition experiments, culture supernatants (diluted 1:10) were pre-incubated with 5 μg of the corresponding peptides overnight at 4 °C in a continuous flow of 5 μl/min in freshly sterilized and degassed HBS buffer pH 7.4 (10 mM-HEPES, 0.15 M-NaCl, 0.05% P20 and 10 μM-ZnCl<sub>2</sub>). NCp7 was immobilized via primary amine groups (Lofas & Johnson, 1990; Malmquist, 1993). The experimental conditions for immobilization (activation time, peptide concentration, pH and ionic strength) were optimized for covalent linking of 500-1000 RU NCp7. Unreacted NHS ester was de-activated by adding 1 M-ethanolamine.

**ELISA.** Antibody purity was determined by antibody reactivity against NCp7 was studied by passing 25 μl crude culture supernatant (or ascitic fluid diluted 1:100 in HBS buffer) over the immobilized NCp7 for 5 min. Dissoication of the immune complexes was followed by a wash with HBS buffer at constant flow rate for 20 min, and the apparent dissociation rate constant was estimated for each MAb reaching saturation binding, as described by Karlsson (Karlsson et al., 1991). The surface was regenerated by injecting 100 mM-HCl and/or 6 M-guanidine chloride pH 7.0 for 3 min.

Inhibition experiments were performed by probing immobilized NCp7 or NCp15 with MAbs either alone or after pre-incubation (2 h at 25 °C) with 10 μg/ml NC peptides (the same set as that used in ELISA). The inhibition caused by a peptide was calculated by comparing the binding of a particular MAb to NCp7 with and without peptide.

**Pairwise mapping.** A saturating amount of the primary MAb (45 μl ascitic fluid diluted 1:50) was injected over NCp7 immobilized on the sensor surface, followed by a second injection of either the same MAb as a control, or another MAb. Each MAb was tested both as primary antibody and, in reverse order, as secondary antibody, for a total of 324 cycles. The sensor surface was regenerated with 15 μl 100 mM-HCl.

**RNA synthesis in vitro.** Primer [32P]tRNALys was generated by T7 RNA polymerase transcrption in vitro of the pTL9 plasmid DNA digested with BanI (Biolabs) in the presence of [32P]UTP (800 Ci/mmol; Barat et al., 1991). [32P]tRNALys was further purified by electrophoresis on 10% polyacrylamide gels with 7 M-urea in 50 mM-Tris-borate pH 8.3. The [32P]tRNALys in the gel slice was recovered by diffusion, precipitated twice with ethanol and dissolved in sterile double-distilled water (Barat et al., 1991). The sp. act. of tRNA<sub>lys</sub> was about 750 Ci/mmol.

**tRNA<sub>lys</sub>-NCp7 cross-linking experiments.** The NC protein was cross-linked to tRNA<sub>lys</sub> by the method of Barat et al. (1991). NCp7 (1 μg/ml) was pre-incubated with 1 μg of each MAb for 5 min at 25 °C in 20 mM-Tris-HCl pH 7.5, 50 mM-NaCl, 0.2 mM-MgCl<sub>2</sub>, 5 mM-DTT and 1 unit/μl of RNAsin (Promega), in a total volume of 10 μl. [32P]tRNA<sub>lys</sub> (2 ng) was then added to each assay and incubation continued for 10 min. Tubes were exposed to UV light (254 nm) for 10 min for cross-linking. SDS was added to a final concentration of 1% and the reaction products were analysed by SDS-PAGE on a 10% gel. The gel was fixed with 10% (v/v) acetic acid, 45% (v/v) methanol, dried and autoradiographed.

**Results**

**Characterization of the anti-NCp7 MAbs by ELISA**

Eighteen anti-NCp7 MAbs were isolated after a primary screening by ELISA against synthetic NCp7 protein (De Rocquigny et al., 1991). The NCp7 epitopes recognized
### Peptide Mapping of Anti-NCp7 MAbs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>1-72 NCp7</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>29-72 NCp7A</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>1-55 NCp7MN</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>13-64 NCp7C</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>12-53 NCp7G</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>1-72 NCp7B</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
<tr>
<td>13-64 NCp7C'</td>
<td>GQRTGQFNLWTSRFGNL-CONH2</td>
</tr>
</tbody>
</table>

Fig. 1. NCp7 synthetic peptides used for epitope mapping of anti-NCp7 MAbs. Schematic representations of synthetic NCp7-derived peptides corresponding to truncated forms of the protein or to small overlapping peptides are shown. The positions on the NCp7 sequence of the first and last amino acids of the peptides are indicated next to the peptide.

By these MAbs were mapped by ELISA using a set of 16 synthetic peptides (Fig. 1) corresponding to different fragments of NCp7. The peptides were either directly coated onto plastic wells or used as competitors for NCp7 recognition. A total of five groups of anti-NCp7 MAbs were identified (Table 1).

Group I contains one rat MAb (i5B11) reacting with residues 1–14 of the N terminus of NCp7. This MAb
Table 1. Epitope mapping of the anti-NCp7 MAbs using ELISA

<table>
<thead>
<tr>
<th>Peptide†</th>
<th>NCp7</th>
<th>NCp7B</th>
<th>NCp7G</th>
<th>NCp7C</th>
<th>NCp7C'</th>
<th>NCp7MN</th>
<th>NCp7A</th>
<th>1-14</th>
<th>52-67</th>
<th>64-80</th>
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<tr>
<td>Group I</td>
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<td></td>
<td></td>
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<tr>
<td>i5B11</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>Group II</td>
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<tr>
<td>EB5, HH3, HG7, DG8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>Group III</td>
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<tr>
<td>GE4, AD2, EC3, CD2, CA5, FC12, JF11</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
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<td>Group IV</td>
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<td></td>
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<tr>
<td>ED8, AC2, JB7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<tr>
<td>CD9, DF3, BE10</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tbody>
</table>

* MAbs are divided into five groups according to their immunoreactivity against NCp7-derived peptides.
+ , positive reaction; -, negative reaction.
† Small overlapping peptides (Fig. 1) had no reactivity with any of the MAbs and are not shown.

Fig. 2. Binding of the anti-NCp7 MAbs to immobilized NCp7 using the BIAcore. The resonance signal (RU) obtained after binding of the MAb to NCp7 was plotted against time for each MAb. About 0.5 ng/mm² NCp7 was immobilized on a dextran matrix and gave the baseline signal (1). The MAbs, injected over 5 min (2), were from hybridoma cell culture supernatants. After injection (3), the dissociation of the antigen-antibody complex (4) was monitored by washing with HBS buffer (see Methods) for 20 min. The dextran matrix was regenerated with HCl (5). The MAb is identified above each curve.

Table 2. Dissociation of MAbs in the BIAcore*

<table>
<thead>
<tr>
<th>MAb</th>
<th>Dissociation (%)</th>
<th>$K_d$ (1/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH3</td>
<td>Low</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>ED8</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>BE10</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>FC12</td>
<td>Moderate</td>
<td>22</td>
</tr>
<tr>
<td>i5B11</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>JB7</td>
<td>High</td>
<td>70</td>
</tr>
</tbody>
</table>

* Results of BIAcore analysis shown in Fig. 2, calculated according to the manufacturer’s instructions.

reacted with peptides 1–14, NCp7MN, NCp7B and NCp7 but did not recognize the overlapping peptide 8–24 or any other N-terminal-deleted peptides. In agreement with results obtained from an epitope library constructed in the pTEX expression vector (M. Bodéus, unpublished results), the epitope target of i5B11 MAb was residues 5–14.

Group II includes four MAbs reacting with residues 52–67. These MAbs recognized only peptides encompassing this domain (NCp7, NCp7B, NCp7C and peptide 52–67) and did not react with peptides 50–59 or 64–80, which overlapped with peptide 52–67. This epitope was located next to the second zinc finger in a basic region of NCp7. Differences within the group II anti-NCp7 MAbs were revealed using NCp7C' mutated at position 59 (Lys to Ser). This mutation resulted in a 10-fold reduction in the reactivity of EB5, but had no effect on the reactivity of HH3, DG8 and HG7. This indicates that the Lys-59 residue is essential for the recognition of NCp7C' by EB5 MAb, but not by the other group II MAbs. The reactivity of the group II MAbs was not affected by the presence of a D-proline residue at position 31, within the basic linker located between the two zinc finger motives, although this D-proline residue disrupts the three-dimensional structure of NCp7 (H. De Rocquigny, unpublished data).

Group III includes seven MAbs reacting with residues 64–72, at the C terminus of NCp7, (peptide 64–80). These MAbs recognized only peptides encompassing this sequence (NCp7, NCp7A and NCp7B), but not peptides 52–67 or 69–89 which overlap with peptide 64–80.
Thus these three groups of anti-NCp7 MAbs recognize precise and limited continuous epitopes located on both sides of the two zinc finger motives. The reactivity of these MAbs in ELISA against their corresponding epitope was independent of the presence or absence of the zinc finger motif.

In addition to these group I, II and III antibodies, which recognized linear epitopes, MAbs from groups IV and V showed no reactivity towards small overlapping peptides or truncated forms of NCp7; they reacted only with NCp7 without or with the zinc fingers (group IV), and with the complete NCp7 (group V). The group IV MAbs, ED8, AC2 and JB7, reacted with both NCp7 and NCp7B, indicating that the reactivity of these MAbs was independent of the zinc finger. The group V MAbs, CD9, DF3 and BE10, only reacted with the complete NCp7 protein, and did not react with any of the other peptides tested, including NCp7B, which lacked the two zinc fingers. These MAbs appear to require the zinc fingers in order to react with NCp7.

**BIAcore analysis of anti-NCp7 MAb reactivity**

The results of the ELISA epitope mapping were confirmed using BIAcore analysis, with NCp7 immobilized on a dextran matrix. Fig. 2 shows examples of the real-time measurements of several anti-NCp7 MAbs. Individual injection curves are shown in overlay format. At the start of the experiment, a buffer continuously flowed over the NCp7-coated surface and the resonance signal followed the baseline (Fig. 2, area 1 of graph). MAb was then injected over 5 min, during which time its binding was seen as a gradual rise in the resonance signal (area 2 of graph). Differences in the maximum height of the plateau were probably due to differences in antibody concentration in the cell culture supernatant used. When the antibody was replaced with buffer (area 3), the immune complex dissociated (area 4). Regeneration buffer was used to remove the MAb and the signal dropped to its original level (area 5 of graph).

The rates of association (ascending part of the curves)
Table 3. Epitope mapping of the anti-NCp7 MAbs by competition studies with the BIAcore

<table>
<thead>
<tr>
<th>MAb*</th>
<th>1-14</th>
<th>NCp7B</th>
<th>NCp7G</th>
<th>NCp7C</th>
<th>NCp7C'</th>
<th>52-67</th>
<th>64-80</th>
</tr>
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<tbody>
<tr>
<td>Class I</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>i5B11</td>
<td>90</td>
<td>90</td>
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<tr>
<td>Class II</td>
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<tr>
<td>EB5, HH3, DG8</td>
<td>--</td>
<td>&gt; 60</td>
<td>&gt; 75</td>
<td>&gt; 85</td>
<td>&gt; 75</td>
<td>&gt; 60</td>
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<td>Class II</td>
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<tr>
<td>HG7</td>
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<td>56</td>
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<td>Class III</td>
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<td>GE4, AD2, EC3, CD2, CA5, FC12, JF11</td>
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<td>90</td>
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<td>&gt; 90</td>
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<td>&gt; 90</td>
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<tr>
<td>DF3, JB7</td>
<td>--</td>
<td>53</td>
<td>--</td>
<td>--</td>
<td>46</td>
<td>50</td>
<td>--</td>
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<td>Class IV</td>
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<tr>
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<td>&gt; 75</td>
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<td>Class V</td>
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<tr>
<td>BE10</td>
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</table>

* MAbs were pre-incubated with the NCp7 peptides and tested for their ability to recognize the immobilized NCp7.

* The percentage binding inhibition caused by the NCp7 peptides was calculated by comparing the binding to NCp7 with and without competitor peptides.

and dissociation (descending part of the curves) for each MAb were different. The dissociation rates (Table 2) were used to divide the MAbs into three categories: (i) MAbs with little or no dissociation (< 11% MAb removed after a 20 min wash), such as HH3, ED8 and BE10; (ii) moderately dissociating MAbs (20–27%), such as FC12 and i5B11 MAbs and (iii) rapidly dissociating MAbs (45–70%) like JB7 MAb (Table 2). Antibodies belonging to the same group have distinct behaviours and different association/dissociation characteristics.

**Competition experiments using the BIAcore instrument**

The BIAcore was also used to investigate the reactivity of the anti-NCp7 MAbs under conditions where recognition of NC protein was blocked by NCp7-derived peptides, as described in Methods.

Eighteen anti-NCp7 MAbs were injected in a pre-programmed series either alone or after pre-incubation with each of 17 peptides (one control and 16 NCp7 peptides). A total of 306 injection cycles were performed using two NCp7 dextran matrices. Sensorgrams of these peptide competitions were analysed to determine the extent of binding of each MAb with or without peptide. Fig. 3 (a) shows the results for the GE4 MAb (from group III). The binding of this MAb was completely inhibited by pre-incubation with peptide 64–80, its target epitope. There was also complete inhibition after pre-incubation with NCp7 protein. But pre-incubation of GE4 MAb with peptide 52–67 or NCp7C had no effect on MAb binding to immobilized NCp7.

The results of these competition assays are shown in Table 3. Partial inhibitions could be due to incomplete representation of the epitope in the synthetic peptide, or to conformational differences between some synthetic peptides and their corresponding linear epitopes in NCp7. The BIAcore results confirmed the classification of the first three groups of anti-NCp7 MAbs (c.f. MAb groups in Table 1 and MAb classes in Table 3): class I MAb (i5B11) was inhibited by peptides containing the N-terminal sequence (1-14); class II MAbs were inhibited by peptides containing the sequence 52–67; class III MAbs were inhibited by peptide 64–72. However, HG7 MAb had a slightly different pattern of reactivity than the other class II MAbs since it was inhibited only by peptides NCp7C' and 52–67 (Table 3).

By contrast, three of the MAbs from groups IV and V showed significant differences between the two techniques. They did not react with continuous epitopes in ELISA. JB7 (from ELISA group IV) and DF3 (from ELISA group V) behaved much like antibodies from class III in the BIAcore (Table 1 and Table 3). Their reactivity with NCp7 was completely or partially inhibited by peptides 64–80, NCp7A and NCp7B. Consequently, JB7 and DF3 MAbs should be considered as a subclass of class III antibodies. These MAbs did not react with peptide 64–80 in ELISA. This suggests that these MAbs were only partially directed against the continuous epitope 64–80, and could react with ad-
HIV-1 NCp7 epitope mapping

Additional NCp7 conformational determinants. CD9 MAb was inhibited by peptide NCp7B in the BIAcore and could be considered to be a class IV antibody, able to react with both forms of NCp7. Three of the MAbs in class IV (ED8, AC2 and CD9) reacted with the entire NCp7 and with the NCp7 lacking the zinc fingers (NCp7B).

Despite these differences between the two techniques, the BIAcore also revealed two types of antibodies that did not react with continuous epitopes and consequently were not inhibited by any of the small overlapping peptides. These MAbs can be distinguished by their behaviour with intact NCp7 and NCp7B. One class V MAb, BE10, recognized only intact NCp7 and did not react with NCp7B (Table 3).

The BIAcore experiments therefore confirmed five different anti-NCp7 MAb classes according to their epitope reactivity, with MAbs specific for linear epitopes (classes I, II and III) or non-linear epitopes (classes IV and V) (Table 3; Fig. 3b).

Recognition of NCp15 by anti-NCp7 MAbs

The BIAcore was used to determine whether the anti-NCp7 MAbs reacted with NCp15, the immediate Gag precursor of NCp7 (Di Marzo Veronese et al., 1987). Class II and III MAbs (HH3 and FC12, respectively) bound to immobilized NCp15 (Fig. 5). This binding was prevented by pre-incubating the antibodies with NCp7 (data not shown). But class IV (ED8) and V (BE10) MAbs did not recognize NCp15, although they bound as efficiently as FC12 and HH3 to immobilized NCp7 (Fig. 1). These results were confirmed using NCp15 in ELISA, where i5B11 reacted with NCp15. Therefore, anti-NCp7 MAbs fall into two categories according to their behaviour towards NCp15: (i) MAbs directed against linear epitopes, like i5B11, FC12 and HH3 MAbs, which reacted with NCp15. This was indeed the case for all the antibodies directed against linear epitopes (data not shown); (ii) MAbs directed against conformational epitopes of NCp7, like BE10 and ED8, as well as AC2.
Inhibition of the tight interaction between NCp7 and HIV-1 primer tRNA\textsubscript{Lys,3} by anti-NCp7 MAbs

NCp7 protein tightly binds to the replication primer tRNA\textsubscript{Lys,3} and this appears to be necessary for the annealing of tRNA\textsubscript{Lys,3} to the primer binding site at the 5' end of the viral RNA (Darlix et al., 1990; Barat et al., 1989). The tight binding of tRNA\textsubscript{Lys,3} to NCp7 was studied using UV irradiation (252 nm), since it promotes cross-linking only when reactive groups are no more than 0.1 nm apart. UV irradiation of the NCp7-tRNA\textsubscript{Lys,3} complexes resulted in the cross-linking of NC protein to tRNA in a 1:1 molar ratio (Fig. 6, lane 2). \[^{32}P\]tRNA\textsubscript{Lys,3} cross-linking to NC protein was not inhibited when unrelated tRNA (yeast tRNA; Boehringer Mannheim) was used as competitor. By contrast, as previously described for tRNA binding to NCp7 (Lapadat-Talposky et al., 1993), cross-linking of \[^{32}P\]tRNA\textsubscript{Lys,3} to NCp7 was abolished when 100- to 500-fold excess of unlabelled tRNA\textsubscript{Lys,3} was used as cold competitor (data not shown). The capacity of MAbs to inhibit the NCp7-tRNA\textsubscript{Lys,3} interactions was determined by pre-incubating NCp7 with each of the 18 MAbs, and analysing its ability to form cross-linked complexes with the tRNA\textsubscript{Lys,3} during UV irradiation (Fig. 6, lanes 3 to 22). The anti-NCp7 MAbs displayed three types of behaviour: (i) nine MAbs did not inhibit the NCp7-tRNA\textsubscript{Lys,3} interactions (HH3, BE10, JF11, HG7, CA5, EC3, CD2, GE4 and EB5; lanes 4, 8, 13, 15, 17, 19, 20, 21 and 22, respectively). Apart from BE10, all these MAbs are directed against linear epitopes of NCp7. One antibody, HH3, enhanced protein-tRNA\textsubscript{Lys,3} interaction by about 40%; (ii) five MAbs inhibited the NCp7-tRNA\textsubscript{Lys,3} interactions by 90% or more (AD2, ED8, CD9, AC2 and JB7; lanes 6, 9, 12, 14 and 18, respectively). It should be noted that apart from AD2, these inhibitory antibodies are directed either uniquely (all MAbs from class IV: ED8, CD9 and AC2) or partially (JB7) against conformational epitopes; (iii) four MAbs partially, but significantly, inhibited the interaction (i5B11, DG8, FC12 and DF3; lanes 3, 5, 7 and 16, respectively). The MAbs that strongly inhibited the NCp7-tRNA\textsubscript{Lys,3} interactions appeared to recognize non-linear epitopes (Table 1). Some MAbs directed against a linear epitope and belonging to one of three different classes (i5B11 of class I, DG8 of class II, AD2 and FC12 of class III) also showed significant inhibitory activity.

Discussion

The HIV-1 NC protein, NCp7, plays a key role in virion assembly and proviral DNA synthesis (Allain et al., 1994; Darlix et al., 1993). We therefore generated MAbs directed against this small viral protein.

A total of 18 anti-NCp7 MAbs were generated and characterized by ELISA and the BIAcore, using direct binding of a set of NCp7-derived peptides, and competition experiments with these peptides. The BIAcore classification (Table 3) is in good agreement with the ELISA epitope mapping (Table 1). Although there are some differences between the two techniques for three of the 18 anti-NCp7 MAbs studied, the MAbs were divided into five classes. Class I contains one MAb which recognizes the N-terminal part of NCp7 (residues 1-14); class II contains four MAbs specific for a basic region of the protein lying next to the second zinc finger (residues 52-67); class III includes nine MAbs which recognize the C-terminal end of NCp7 (residues 64-72). The MAbs in these three classes are directed against continuous and linear epitopes. Three MAbs from class IV (ED8, CD9 and AC2) and one MAb from class V (BE10) are specific for non-continuous epitopes; they showed no reactivity against any of the small overlapping peptides tested. MAbs from class IV react with both NCp7 and NCp7 lacking zinc fingers. Clearly, these antibodies do not require the zinc fingers to react with NCp7. But their reactivity depends on the presence of the N- and C-
terminal parts of the protein as well as the linker domain located between the two zinc fingers. The class V MAb, BE10, reacts only with whole NCp7, and not with NCp7 lacking zinc fingers. This antibody seems to be dependent on the presence of the zinc fingers in order to react with NCp7. However, BE10 MAb does not react with peptides 13–55 or 13–64, which are the two zinc fingers without the flanking N- and C-terminal domains.

Thus, class IV and V anti-NCp7 MAbs are directed against non-linear and conformational epitopes. DF3 and JB7 MAbs, from a subclass of class III, are inhibited by peptide 64–80 in the BiACore, but they do not react with this peptide in ELISA. This suggests that these MAbs are only partially directed against the continuous epitope 64–80, and could react with additional conformational NCp7 determinants.

BiACore technology was used to study the stability of the immune complexes formed between the MAbs and NCp7. Some MAbs readily dissociated from the protein, whereas others were firmly bound.

The anti-NCp7 MAbs were also assayed for their ability to recognize NCp15, the immediate precursor of NCp7. Classes I, II and III MAbs, which are specific for continuous epitopes, interacted with immobilized NCp15. Class IV and V MAbs, which are specific for discontinuous epitopes, did not react with NCp15. Previous observations have shown that: (i) HPLC-purified synthetic NCp7 (more than 99% pure) displays full biological activity in vitro (De Rocquigny et al., 1991, 1992); (ii) ED8 (class IV MAb), in contrast to MAbs from class I and II, can recognize Pr55Gag only in non-denaturing conditions (Tanchou et al., 1994); (iii) the anti-NCp7 MAbs react with purified NCp7 from virions (Ottmann et al., 1995) or from retrovirus-like particles produced in baculovirus (Carrière et al., 1995). These observations, taken together with the results of this study, suggest that the N-terminal 72 amino acids of NCp15 that form the NCp7 moiety are probably folded into a tertiary structure different from that of mature NCp7. These results are also the first evidence of conformational changes between the precursor and the mature form of the NC protein of HIV-1. The three-dimensional structure of NCp7 has been elucidated by NMR (Morellet et al., 1992), and NMR or crystallography studies on the structure of NCp15 are needed to characterize the structural changes responsible for the differences observed in the immunoreactivity of the two proteins. Nevertheless, these results showed that the anti-NCp7 MAbs can be used as conformational probes to study the NCp7 protein in the course of virion assembly.

Lastly, we determined whether the anti-NCp7 MAbs inhibited the tight binding of the replication primer tRNA^lys,3 to NCp7. Nine of 18 MAbs inhibited this NCp7–tRNA^lys,3 interaction. These results suggest that the specific inhibition caused by these nine MAbs (Fig. 6) is due to recognition of their target epitopes, rather than to non-specific steric hindrance caused by the binding of an antibody molecule 20 times bigger than NCp7 itself. Binding of the inhibitory MAbs to their target epitope could also induce a conformational change in NCp7, or cause steric hindrance impairing the tRNA binding to NCp7.

The strongest inhibitors of the NCp7–tRNA^lys,3 interaction were MAbs directed either exclusively (ED8, CD9 and AC2) or partially (JB7) against non-linear epitopes (Table 1). Further studies using site-directed mutagenesis should allow us to identify the amino acids involved in the recognition of NCp7 by these MAbs, and consequently the amino acids needed for the NCp7–tRNA^lys,3 interaction. Some anti-NCp7 MAbs directed against linear epitopes were also able to partially (i5B11, DG8 and FC12) or totally (AD2) inhibit this interaction. This suggests that these MAbs, unlike the other members of their respective classes which have no inhibitory activity, probably recognize NCp7 residues involved, at least in part, in the tight binding of NCp7 to tRNA^lys,3.

In fact, most of the MAbs directed against linear epitopes and which react with NCp15, the precursor of NCp7, are unable to inhibit in vitro the NCp7–tRNA^lys,3 tight binding. Further studies using NCp7 mutants and peptides should identify the amino acids involved in the NCp7–tRNA^lys,3 interactions in vitro. Last, but not least, this library of 18 anti-NCp7 MAbs will be used to analyse the structure of the virion NC in the course of virus budding and entry into a target cell.

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