Characterization of a discontinuous epitope of the human immunodeficiency virus (HIV) core protein p24 by epitope excision and differential chemical modification followed by mass spectrometric peptide mapping analysis

ELISABETH O. HOCHLEITNER, CHRISTOPH BORCHERS, CAROL PARKER,
RACHELLE J. BIENSTOCK, AND KENNETH B. TOMER

National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

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Abstract

A combination of epitope excision, epitope extraction, and differential chemical modification followed by mass spectrometric peptide mapping was used for the characterization of a discontinuous epitope that is recognized by the mouse anti-HIV-p24 monoclonal antibody 5E2.A3. In epitope excision, the protein is first bound to an immobilized antibody and then digested with proteolytic enzymes. In epitope extraction, the protein is first digested and subsequently allowed to react with the antibody. After epitope excision of the p24-5E2.A3 complex with endoproteinase Lys-C, a large fragment remained affinity bound corresponding to amino acids 1–158 of HIV-p24 (fragment 1–158). Further digestion, however, resulted in loss of affinity. Moreover, no affinity-bound fragments were observed after an epitope extraction experiment. These data from the epitope excision and extraction experiments suggest that the epitope is discontinuous. For the further characterization of the epitope, amino groups in the epitope-containing fragment were acetylated in both the affinity bound and free states followed by mass spectrometric analysis. Two successive acetylation reactions were performed: (1) the first used a low molar excess of acetic anhydride, and (2) the second, after separation from the antibody, a high molar excess of its hexadeuteroderivative. This isotopic labeling procedure, in combination with high resolution mass spectrometry, allowed the precise determination of relative reactivities of amino groups. In this study, no differences were observed in the ranking of the relative reactivities of five lysine residues. However, the N-terminal amino group was found to be part of the discontinuous epitope.

Keywords: acetylation; differential chemical modification; discontinuous epitope; HIV-p24; mass spectrometry

The human immunodeficiency virus (HIV) *gag* gene encodes a 55 kDa polyprotein that is a precursor of the proteins of the viral core. During budding of the virus from the host membrane, the *gag* polyprotein is cleaved by a virus-encoded protease (Debouck et al., 1987), resulting in the HIV proteins p24, p17, p7, and p6. The protein HIV-p24 consists of 231 amino acids and is the major component of the inner core of HIV (Gelderblom et al., 1987). It forms a conical shell around the RNA genome and the nucleocapsid protein HIV-p7. HIV-p24 and its 55 kDa precursor play a crucial role in the assembly, maturation and disassembly of HIV (Reicin et al., 1995, 1996).

Screening procedures for HIV-antigens (Healey et al., 1988) and anti-HIV antibodies (Vasudevachari et al., 1989) are used to detect the presence of HIV infection in serum samples. The possibility of

testing for the presence of HIV-p24 occurs within a window of about six weeks between infection and seroconversion, where a test for the presence of anti-HIV antibodies is not effective. A test for HIV-p24 will probably have to be combined with a test for antibodies (Gurtler et al., 1998), since HIV-p24 usually cannot be detected after seroconversion, due to strong humoral and cell mediated immune responses (Gaines et al., 1987; Chou et al., 1988; Janvier et al., 1996). Intense interest has also been focused on generating antibodies against conserved regions of HIV-p24, which maintain their affinity despite the virus's high mutation rate (Watkins et al., 1996). Elucidation of the antigenic structure of HIV-p24 is of importance to increase the reliability and the sensitivity of HIV screening procedures and possibly may be useful in the development of HIV vaccines.

Antigenic epitopes can consist of linear stretches of amino acids or discontinuous amino acids that are in proximity due to the conformation of the protein. Several methods have been described for the characterization of epitopes, such as X-ray analysis, pro-

Reprint requests to: Kenneth B. Tomer, National Institute of Environmental Health Sciences, National Institutes of Health P.O. Box 12233, Research Triangle Park, North Carolina 27709; e-mail: tomer@niehs.nih.gov.

teolytic footprinting, and epitope excision/extraction techniques. X-ray crystallography has been successfully used for the characterization of continuous and discontinuous epitopes; however, this method is time consuming and requires large amounts of sample (Davies & Cohen, 1996; Keitel et al., 1997). Proteolytic footprinting is based on the protection of residues in the antigen that are involved in affinity binding against proteolysis or chemical modification. An important feature of this method is that the antibody shows high resistance to proteolytic digestion (Jemmerson & Paterson, 1986; Burnens et al., 1987). Recently, epitope excision/epitope extraction methods in combination with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry were used for the characterization of linear epitopes in the proteins HIV-p24 and HIV-gp120 (Parker et al., 1996; Jeyarajah et al., 1998). In epitope excision, the protein in its native structure is bound to the immobilized antibody and is subsequently digested with endoproteolytic enzymes. In the epitope extraction method, the protein of interest is first digested with endoproteases and then incubated with the immobilized antibody. The applicability of these methods for the characterization of discontinuous epitopes, however, can be limited. Often, only binding of large fragments is observed and further enzymatic cleavage between the residues composing the epitope leads to a loss of affinity. Recently, a discontinuous epitope in hen egg white lysozyme has been characterized by differential chemical modification of amino acids in combination with mass spectrometry (Fiedler et al., 1998). The concept behind this method is that the reactivity of amino acid residues in a protein correlates to their surface accessibility (Glocker et al., 1994). Thus, a comparison of the reactivity of the amino acid residues in the free antigen and of the residues in the antigen bound to the antibody in its native conformation can provide information about which amino acids are involved in paratope-epitope interaction.

We describe here the characterization of a conformational epitope recognized by the mouse anti-HIV-p24 monoclonal antibody 5E2.A3 by epitope excision, epitope extraction, and differential chemical modification of lysine residues by acetylation followed by mass spectrometric peptide mapping. The fragment, which remained affinity bound after epitope excision, was treated with two successive acetylation reactions: first with a low molar excess of acetic anhydride, and after separation of the fragment from the antibody, with a high molar excess of hexadeuteroacetic anhydride. This isotopic labeling procedure followed by mass spectrometry allows the precise determination of relative reactivities of lysine residues in the affinity bound and free protein by determination of the abundance ratio of the monoisotopic ion signals from the corresponding acetylated and trideuteroacetylated peptides.

Results

Epitope excision and extraction

The monoclonal antibody 5E2.A3 was immobilized on CNBr Sepharose beads and incubated with HIV-p24 (strain SF-2). The beads with the affinity-bound protein were analyzed directly by MALDI-TOF mass spectrometry. The molecular mass of affinity bound HIV-p24 was found to be 25,563 Da (data not shown), which agrees well with the theoretical average mass of 25,565 Da calculated from the amino acid sequence of HIV-p24. No affinity-bound proteins were detected in the absence of HIV-p24. These results

Fig. 1. MALDI-TOF mass spectrum (acquired in a mass range of 500–30,000 Da) of the fragment 1–158 that remained affinity bound after digestion of the HIV-p24/5E2.A3 complex with endoproteinase Lys-C for 12 h ($[M+H]^+$ 17,462).

demonstrate that HIV-p24 (1) binds to the 5E2.A3 antibody and (2) that MALDI-TOF mass spectrometric analysis is highly suited to determine the molecular weight of antigens bound to antibodies immobilized on CNBr Sepharose beads.

After digesting the affinity bound protein with endoproteinase Lys-C overnight and analysis by MALDI-TOF mass spectrometry, only one fragment with an m/z of 17,462 Da was detected, which corresponded to the singly charged N-terminal fragment of HIV-p24 (amino acids 1–158) with a theoretical average MW of 17,458 Da (Figs. 1, 2). An aliquot of this suspension was then digested with endoproteinase Lys-C for a further 12 h. No peptides, however, were detected after the second digest indicating that no protein remained bound to the antibody. Digestion of the

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1
PIVQNLOGQMVHQAI SPRTLNAWVKVVEEKAFSPEVIPMF
41
SALSEGATPQDLNTMLNTVGGHQAMQMLKETINEEAAEW
81
DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTN
121
NPPIPVGEIYKRWIILGLNKKIVRMYSPSILDIRQGPKEP
161
FRDYVDRFYKTLRAEQASQDVKNWMTETLLVQNANPDCKT
201
ILKALGPAATLEEMMTACQGVGGPGHKARVL

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Fig. 2. Amino acid sequence of intact HIV-p24 and of the fragment of HIV-p24 that remains affinity bound after Lys-C digestion of the immune complex for 12 h (underlined).

affinity bound Lys-C fragment with endoproteinase Glu-C for 12 h or trypsin for 6 h resulted in a loss of affinity. The peptides found in the supernatant were identical to those observed for the corresponding reactions of the unbound HIV-p24 fragment 1–158 (data not shown). These data suggest that the epitope in HIV-p24 recognized by the 5E2.A3 antibody is discontinuous. An epitope extraction experiment was performed to further confirm this hypothesis. In this experiment, a Lys-C digest of HIV-p24 was added to immobilized 5E2.A3 antibody beads and incubated for 2 h. No affinity bound peptides were observed by MALDI-TOF analysis (data not shown). These data are consistent with the conclusion from the epitope excision experiment that this epitope is discontinuous.

Acetylation of the side-chain amino groups in free and affinity bound HIV-p24

To obtain further information about the residues in HIV-p24 involved in the interaction with the 5E2.A3 antibody, lysine residues in the affinity bound and free fragment 1–158 were modified with acetic anhydride. Free amino groups in proteins are highly sensitive to their chemical environments. Their reactivity can be influenced by factors that alter their pK_a or change their surface accessibility. Using several model proteins, it has been demonstrated that the reactivity of the ϵ -amino group of lysine residues correlates directly with their surface accessibilities (Suckau et al., 1992; Glocker et al., 1994). Figure 3 depicts the strategy used for

Fig. 3. Analytical scheme of the differential chemical modification of amino groups in fragment 1–158 of HIV-p24 with acetic anhydride. Affinity bound and free protein are first treated with a 10,000-fold molar excess of acetic anhydride followed by separation from the antibody, and reaction with a 100,000-fold molar excess of hexadeuteroacetic anhydride. Relative reactivities are calculated from the ratio of the intensities of the acetic and trideuteroacetylated peptides. Acetylation of two lysine residues is illustrated. The lysine residue in peptide 2 has similar reactivity in affinity bound and free antigen. The lysine residue in peptide 1 is involved in affinity binding and has a reduced reactivity in the immune complex.

the identification of residues involved in affinity binding. In the first acetylation reaction, a lower molar excess of acetic anhydride is used for the partial acetylation of the affinity bound antigen. Subsequently, the antibody–antigen complex is separated and purified by high-performance liquid chromatography (HPLC). Then, a second acetylation reaction of the antigen is performed with a high molar excess of the hexadeuteroderivative of acetic anhydride, and the antigen is again purified by HPLC. As a control, the unbound antigen is identically treated. The control reaction serves to take into account variations in the reactivities of the ϵ -amino groups in the HIV-p24 fragment due to the presence of ion pairs or other intramolecular interactions. The two samples are digested with endoproteases, which results in a mixture of acetylated and trideuteroacetylated derivatives of the same peptides. These peptides are then separated and analyzed by liquid chromatography/mass spectrometry (LC-MS) and liquid chromatography/tandem mass spectrometry (LC-MS/MS). Acetylated and trideuteroacetylated peptides are identified from the resulting chromatogram based on their mass shifts of 42 and 45 Da, respectively. The relative reactivity of the amino groups in the affinity bound and free antigen is calculated from the ratio of the intensities of the mono-isotopic ion signals of the acetylated and trideuteroacetylated peptides. Comparison of the relative reactivities in the immune complex and the unbound antigen can provide information about which residues are involved in binding to the antibody.

As a first step, the molar excess of acetic anhydride, relative to HIV-p24, which is sufficient for the partial and complete acetylation of amino groups in the affinity bound fragment 1–158 was determined. The antibody–antigen complex was treated in solution with increasing molar excess of acetic anhydride. The acetylation reaction was performed at neutral pH, so that the native conformation of HIV-p24 was retained. The reaction time was constant and therefore the extent of acetylation depended mainly on the molar excess of acetic anhydride. The acetylated fragment 1–158 was subsequently separated from the antibody and purified by HPLC, and the acetylated fragment was identified based on molecular weight by MALDI-TOF mass spectrometry (data not shown). Acetylation of one amino group in a protein results in an increase of the molecular weight by 42 Da. Figure 4 shows the correlation between the average number of the acetylated amino groups calculated from the increase in molecular weight of the affinity bound N-terminal fragment 1–158 and the molar excess of acetic anhydride applied.

Fig. 4. Correlation of the average number of acetylated amino groups to molar excess of acetic anhydride for the affinity bound fragment 1–158 of HIV-p24. The molecular weight of the singly charged acetylated fragment 1–158 was determined using MALDI-TOF mass spectrometry.

The curve has a hyperbolic shape and reaches a plateau at a m/z of 17,746 Da, which corresponds to the incorporation of seven acetyl groups. This is consistent with the number of amino groups in the N-terminal fragment 1–158 and emphasizes the high specificity of the acetylation reaction for these groups. The high molar excess needed for all reactions can be explained by the low concentration of HIV-p24 ($0.1 \mu\text{g}/\mu\text{L}$) favoring side reactions (hydrolysis) of acetic anhydride.

For the determination of the relative reactivities of the lysine residues, the affinity bound fragment 1–158 was first partially acetylated with a 10,000-fold molar excess of acetic anhydride. With a 10,000 molar excess of acetic anhydride, five lysine residues are acetylated in average in the affinity bound fragment 1–158. Subsequently, the acetylated fragment 1–158 was purified and separated from 5E2.A3 antibody by HPLC. The acetylated fragment 1–158 was identified based on molecular weight by MALDI-TOF mass spectrometry. Fragment 1–158 of HIV-p24 was acetylated completely with a 100,000 molar excess of hexadeuteroacetic anhydride. In the control experiment, unbound fragment 1–158 of HIV-p24 was treated identically. The relatively high excess used in the first acetylation reaction assures the partial acetylation of all lysine residues in fragment 1–158 of HIV-p24. To test whether the antibody/antigen complex remains intact during the first acetylation reaction, fragment 1–158, acetylated with a 10,000-fold molar excess of acetic anhydride, was added to immobilized 5E2.A3 antibody. The beads were washed with phosphate buffered saline (PBS), and an aliquot was analyzed with MALDI-TOF mass spectrometry. The acetylated fragment 1–158 was identified based on molecular weight and was found to bind to the antibody (data not shown). This indicates that partial acetylation of the lysine residues in the fragment 1–158 did not significantly change the tertiary structure of the epitope region. Ehrhard et al. (1996) modified the lysine residues in HIV-p24 with maleic anhydride and analyzed the conformation and stability of unmodified and highly modified HIV-p24 (9 of 12 amino groups modified in average) by circular dichroism and fluorescence spectroscopy. Only minor differences in the conformation of the highly modified and unmodified protein were observed. The subsequent acetylation of fragment 1–158 with hexadeuteroacetic anhydride was performed in the absence of the antibody. Because the lysine residues are fully acetylated (Fig. 4), potential conformational changes during the complete acetylation should not change the ratio of the resulting acetylated and trideuteroacetylated peptides.

The acetylated/trideuteroacetylated fragment 1–158 of HIV-p24 was digested with trypsin and the resulting peptides were analyzed by LC-MS and LC-MS/MS. Since tryptic digestion is inhibited when lysines are acetylated, this results in multiply-acetylated peptides, necessitating the further digestion of the tryptic digest of fragment 1–158 with endoprotease Glu-C overnight. Figure 5A shows the LC-MS chromatogram of a tryptic and subsequent Glu-C digest of the acetylated fragment 1–158 of HIV-p24. To facilitate the identification of acetylated and trideuteroacetylated peptides precursor ion scanning was performed as described previously (Borchers et al., 1999). Scans for the parents of common fragmentation products of peptides, such as immonium ions, can improve the sensitivity for the identification of peptides (Wilm et al., 1996). In contrast to triple quadrupole precursor ion scanning, precursor ion scanning with the Q-TOF involves acquisition of complete MS/MS spectra from a selected m/z range of potential precursors. The MS/MS data are then searched for the presence of the fragment ion of interest (Borchers et al., 1999). The extracted LC-

Fig. 5. A: Total ion current (TIC) chromatogram of a tryptic and subsequent Glu-C digest of fragment 1–158 of HIV-p24. B: The reconstructed ion chromatograms after precursor ion scanning for the immonium ion of acetyllysine (m/z 143). C: The immonium ion of trideuteroacetyllysine (m/z 146).

MS/MS chromatograms for the immonium ions of the acetylated (m/z 143; Fig. 5B) and the trideuteroacetylated (m/z 146; Fig. 5C) peptides were generated. Figures 5B and 5C are extracted from one of four LC-MS/MS precursor scans. The peak ($[M + 2H]^{2+} = 421.34/422.85$) at a retention time of ~ 57 min was found to contain both acetylated and trideuteroacetylated residues. Based on sequence data acquired by MS/MS, this peptide was found to correspond to the acetylated/trideuteroacetylated peptide 137–143 (MW: 840.51/843.51 Da) of HIV-p24 (Fig. 6). Other acetylated peptides were also identified using this method.

Acetylated/trideuteroacetylated peptides were identified that contained the acetylated amino acids P-1, K-25, K-30, K-140, and

K-158. These were identified based on the observation of mono-acetylated tryptic fragments, amino acids 1–18, 19–29, 30–35, 133–143, and 155–158, respectively. A monoacetylated peptide with unusual tryptic cleavage sites, amino acids 55–71, was found to contain modified K-70 and to be due to cleavage by chymotrypsin, which is a contamination of the trypsin preparation. A second identified peptide containing modified K-140 (amino acids 137–143) was also found to be produced by tryptic and chymotryptic cleavage. No ions corresponding to K-131 were found in the LC-MS chromatograms, which may be due to the low pI (4.6) of this tryptic peptide. Acidic peptides have been shown to generate lower ion currents in the mass spectrometer; a similar effect has been observed for phosphopeptides (Asara & Allison, 1998).

Table 1 shows the rankings of the relative reactivities of the lysine residues in the affinity bound and free N-terminal fragment 1–158. The rankings were compared instead of individual relative reactivity values, because the latter can vary due to experimental differences or conformational changes of the HIV-p24 fragment induced by binding of the antibody. Crystal structures of antibody Fab fragments and their bound antigens revealed that binding of the antibody to the antigen may be accompanied by conformational changes in both the antibody and the antigen. However, structural information for only a few antibody–antigen complexes is presently available and a major part of the available information comes from studies of antibody complexes with small antigens (Davies & Cohen, 1996; Jackson, 1999).

Conformational changes could also be caused by low pH conditions during the HPLC isolation of the unbound HIV-p24 fragment; these potential conformational changes should be reversible, however, on return to neutral pH as shown by Misselwitz et al. (1995). The ranking of the relative reactivities of the amino groups

Fig. 6. Tandem mass spectrum of the doubly charged ion ($[M + 2H^{2+}]$ 421.34) of the acetylated peptide with the sequence GLNKIVR (amino acids 137–143) of HIV-p24. The doublet due to the presence of acetyl (d_0) and trideutero (d_3) acetyllysine is enlarged. Fragment ions denoted by an asterisk (*) contain the d_0/d_3 acetyllysine residue.

in the unbound fragment 1–158 of HIV-p24 determined in this study is comparable to the ranking observed upon acetylation of the intact HIV-p24 (Borchers et al., 1997; C. Borchers, R.J. Bienstock, X.-H. Xian, & K.B. Tomer, unpubl. obs.), demonstrating that conformational changes during HPLC are negligible.

The rankings of the relative reactivities of lysines 25, 30, 70, 140, and 158 of the free and affinity bound fragment 1–158 of HIV-p24 exhibit no differences. The N-terminal amino group, however, has highly reduced reactivity to acetylation in the immune complex (Fig. 7A). The low reactivity of Pro-1 suggests that this amino acid is involved in affinity binding to the 5E2.A3 antibody, whereas lysine residues 25, 30, 70, 140, and 158 do not seem to be located within the epitope structure. Although it is tempting to ascribe the minor changes in the reactivity of lysines 30, 70, 140, and 158 to slight conformational changes in the HIV-p24 fragment, when bound to the antibody, insufficient experimental data are presently available to distinguish this possibility from slight variations in experimental conditions. The fact that only major differences in the rankings are observed for Pro-1 may rule out large conformational changes in the fragment 1–158 of HIV-24, induced by binding of 5E2.A3 antibody. Figure 8 shows the NMR structure of truncated HIV-p24 (amino acids 1–151) described by Gitti et al. (1996). The N-terminal proline (yellow) and lysines 25, 30, 70, and 140 (turquoise) are highlighted. The average epitope surface, which can be covered by an antibody, has been estimated from X-ray analyses of crystallized antibody–antigen complexes to be $\sim 700 \text{ \AA}^2$ (Davies & Cohen, 1996). Assuming that Pro-1 is located in the center of the epitope area, residues within a radius of $14 \text{ \AA}$ should also be shielded from amino acetylation. This hypothetical epitope surface is marked in magenta in Figure 8. Lysine residues 25, 30, 70, and 140 are not located within this epitope surface, indicating that the distances of these residues to Pro-1 are greater than $14 \text{ \AA}$. This explains why the ranking of the relative reactivities of the lysine residues is not affected by binding to the antibody.

Discussion

Epitope excision and extraction methods in combination with MALDI-TOF mass spectrometry allow the rapid characterization of linear epitopes. When the epitope is discontinuous, however, often only binding of large fragments is observed and affinity can be lost due to enzymatic cleavage between the amino acids in-

involved in binding. For this reason, we combined these methods with differential chemical modification of lysine residues with acetic anhydride followed by mass spectrometry. Since the epitope excision experiments already provide information about the region involved in affinity binding, analysis of a fragment instead of the entire protein reduces the time and the complexity of the analytical problem. Isotopic labeling of lysine residues with two successive acetylation reactions in combination with mass spectrometry with high resolution allows the precise determination of relative reactivity data. Recently, the role of lysine residues in the DNA binding of *Escherichia coli* single strand binding protein was determined using this isotopic labeling technique (Chen et al., 1998). In that study, a low molar excess of hexadeuteroacetic anhydride was used in the first reaction, and subsequently a high molar excess of acetic anhydride followed by mass spectrometric peptide mapping. Errors, however, can be made in the determination of the relative reactivities, due to overlapping of the acetylated ion containing three ^{13}C atoms and the corresponding monoisotopic trideuteroacetylated ion signal of the peptide. For example, for the singly charged tryptic fragment comprising amino acids 1–18 of HIV-p24 (m/z 2,016), the relative abundance of the isotopic ion containing three ^{13}C atoms to the monoisotopic ion is $\sim 32\%$. For this reason, we used a low molar excess of acetic anhydride in the first acetylation reaction followed by a high molar excess of hexadeuteroacetic anhydride. This minimizes the overlapping of the acetylated and trideuteroacetylated ions of the same peptides, and thus reduces errors made in the determination of the abundance of the trideuteroacetylated peptide ion.

This epitope mapping methodology can be further extended by differential chemical modification of other amino acids (Glazer, 1970), such as modification of arginine residues with 1,2 cyclohexandione or iodination of tyrosine residues (Fiedler et al., 1998). Another possibility to extend this method could be the combination with hydrogen-deuterium exchange followed by mass spectrometry. This approach has recently been applied to study protein–protein and protein–ligand interactions. With this method, differences in the hydrogen exchange rates with and without ligands can be investigated. Reduced exchange rates upon binding of the ligand would provide information about which regions are involved in binding (Wang et al., 1997). The data obtained from the surface modification and hydrogen-deuterium exchange experiments can be supplemented by available structural information from X-ray crystallography or NMR analysis. Molecular modeling studies could also be performed by combining the structural information available with the data obtained from mass spectrometry experiments. Since mass spectrometry data from chemical modification and exchange experiments yield information concerning whether amino acid residue side chains are exposed or buried in a protein, this information might be introduced as a distance constraint in a molecular dynamics modeling study. Studies such as these could be performed to obtain a more complete structural description of proteins that require modification or truncation to be solved by X-ray or NMR methods. An example is the HIV-p24 protein (Borchers et al., 1997), whose structure was initially reported as a truncated 151 residue protein (Gitti et al., 1996).

Conclusion

In this study, we used epitope excision, epitope extraction, and differential chemical modification of lysine residues followed by mass spectrometric peptide mapping for the characterization of the

Table 1. Ranking of relative reactivities of lysines and the N-terminal amino group of (A) free and (B) affinity bound fragment 1–158 of HIV-p24^a

A			B		
Ranking	Amino acid	Relative reactivity	Ranking	Amino acid	Relative reactivity
1	Pro1	3.3	1	Lys158	2.3
2	Lys158	1.7	2	Lys30, 70	1.9
3	Lys30, 70	1.1	3	Lys140	0.70
4	Lys140	1.0	4	Lys25	0.60
5	Lys25	0.60	5	Pro1	0.31

^aThe variation of the relative reactivities calculated from two runs of the same sample was 2–3%.

Fig. 7. LC-MS spectrum of the tryptic peptide comprised of amino acids 1–18 containing acetylated and trideuteroacetylated Pro1 of the (A) affinity bound and (B) free fragment 1–158 of HIV-p24. The isotopic peaks at m/z 1,029.61 and m/z 1,031.16 correspond to the doubly charged acetylated (+42 Da) and trideuteroacetylated peptide (+45 Da) forms of the peptide, respectively.

epitope recognized by the anti-HIV-p24 mouse monoclonal antibody 5E2.A3. The epitope of the 5E2.A3 monoclonal antibody was found to be discontinuous and includes the N-terminal proline residue, which is highly conserved among various HIV-1, HIV-2, and simian immunodeficiency virus (SIV) isolates. The cross reactivity of the antibody in ELISA with the HIV-2 and SIV core proteins p26 and p27 can now be explained by the high conservation of Pro-1. The use of this antibody may have potential in the screening of blood samples for HIV. Since this antibody is not only specific to a conserved region of HIV-1, but cross reacts with HIV-2 and even SIV isolates, its application may contribute to the development of more reliable and safe diagnostic assays.

Materials and methods

Materials

Recombinant HIV gag protein HIV-p24 was purchased from Austral Biologicals (San Ramon, California) and was purified by ion-exchange chromatography followed by gel filtration chromatography. The anti-HIV-p24 mouse monoclonal antibody 5E2.A3 (subclass IgG1), which was obtained from Biodesign International (Kennebunk, Maine), cross reacts with HIV-2 p26 and SIV p27 in ELISA, according to the manufacturer. Endoproteinase Lys-C was obtained from Wako Chemical Co. (Dallas, Texas), trypsin TPCK from Worthington Biochemical Co. (Freehold, New Jersey), and endoproteinase Glu-C from Sigma (St. Louis, Missouri).

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were acquired on a Voyager-RP MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, Massachusetts) equipped with a nitrogen laser ($\lambda = 337$ nm). α -Cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, Wisconsin) was recrystallized in hot methanol, stored in the dark, and prior to sample analysis, a fresh saturated solution of the matrix in water/ethanol/formic acid 45/45/10 (v/v) was prepared. One-half microliter of the sample was mixed with 0.5 μ L of the saturated matrix solution. This solution was then pipetted onto the target and dried at room temperature. One hundred twenty-five laser shots were used per sample generate a single mass spectrum. External calibration was performed with two standard substances bracketing the desired mass range.

Liquid chromatography

For the separation and purification of acetylated proteins, a Hewlett Packard (Wilmington, Delaware) 1100 HPLC system equipped with a Vydak C4 column (4.2 mm i.d., 5 μ m particles), a diode array detector, and a fraction collector was used. Gradient elution was carried out with two solvents: A (water, 0.1% TFA (v/v)) and B (90% acetonitrile, 10% water, 0.09% TFA (v/v)). A linear gradient of 10% B–60% B over 50 min was used to purify the proteins for later analysis.

Fig. 8. NMR structure of HIV-p24 described by Gitti et al. (1996). Pro1 is marked in yellow and lysines are marked in turquoise. The surface area, which can be covered by the 5E2.A3 antibody, supposing that Pro1 is in the center of the epitope, is highlighted in magenta.

Liquid chromatography with mass spectrometric (LC-MS) and tandem mass spectrometric (LC-MS/MS) detection

Capillary liquid chromatography (LC) with mass spectrometric (MS) and tandem mass spectrometric (MS/MS) detection was performed using a Gilson Gradient HPLC (Gilson, Middleton, Wisconsin) connected inline to a Micromass Q-TOF mass spectrometer (Micromass, Altrincham, UK). The initial flow rate of 0.5 mL/min was split precolumn to ~200 nL/min using an LC Packings (San Francisco, California) Acurate® splitter. A 15 cm × 75 μm i.d. fused silica column (Hypersil RP-C18, LC Packings) was used to separate the peptides. It was connected by a length of fused silica capillary (1 m × 50 μm i.d.) to an LC Packings FAMOS autosampler and to the nebulized nanospray source via Teflon connectors. The following LC conditions were used for all separations: solvent A (water, 0.1% formic acid (v/v)), solvent B (acetonitrile, 0.1% formic acid (v/v)), and linear gradient of 5 to 95% B over 30 min. Samples were diluted in 50% acetonitrile/0.1% formic acid. The injection volume used was 2.5 μL. The needle voltage used for all analyses was 2,800 V and the cone voltage was 30 V. The total ion current chromatograms were acquired over the mass range of 400–1,500 Da.

For the acquisition of MS/MS spectra, data dependent switching was used. The instrument performs MS/MS on series of ions, which meet a certain preselected abundance. A family of MS/MS “chromatograms” designated MS/MS 1–4 (corresponding to the most abundant ion in the preceding MS, the second most abundant, etc.) is generated that can be searched for a certain characteristic mass. The MS/MS spectra were acquired over a mass range of 40–2,000 Da with a collision energy of 30 eV. The mass resolution

of MS-I was set to pass the entire isotope cluster (including d_0 and d_3 ion species) of the species of interest. Approximately 50 scans were combined to generate a single spectrum.

Epitope excision and extraction

CNBr-activated Sepharose beads (0.1 g) were suspended in 5 mL of 0.1 M HCl and equilibrated for 15 min. Approximately 100 μL of the beads were pipetted from the bottom of the vial into compact reaction columns. The beads were then washed with 0.1 M HCl followed by 0.1 M NaHCO₃ coupling buffer. The antibody (20 μg) was added to the suspension and incubated for 2 h with slow rotation at room temperature. The beads with the covalently attached antibody were washed with 0.1 M Tris-HCl buffer (pH 8.0). Blocking of unreacted groups on the CNBr Sepharose beads was accomplished by incubation for 2 h with a 0.1 M Tris-HCl buffer (pH 8.0). Uncoupled antibody was removed by sequential washing with two buffers with different pH: first with a 0.1 M sodium acetate, 0.5 M NaCl (pH 4.0) buffer and, subsequently, with a 0.1 M Tris-HCl (pH 8.0) buffer. The beads were then equilibrated in PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) and incubated for 2 h at room temperature with and without HIV-p24. After washing the beads with PBS (pH 7.2), an aliquot of the suspension was removed for MALDI-TOF analysis. Epitope excision and extraction experiments were performed as previously described (Parker et al., 1996). For epitope excision, the affinity bound protein was digested with endoproteinase Lys-C (1:20 enzyme:substrate ratio) in PBS (pH 7.2) at 37 °C with slow rotation for 12 h. Digestions with trypsin or endoproteinase Glu-C

were performed in 50 mM NH_4HCO_3 buffer (pH 7.8) with an enzyme:substrate ratio of 1:20 for 6 and 12 h, respectively. Following proteolysis, the beads were washed in the digestion buffer and 0.5 μL of the suspension removed for MALDI-TOF analysis.

For epitope extraction, HIV-p24 in PBS (pH 7.2) was digested with endoproteinase Lys-C (1:20 enzyme:substrate ratio) at 37 °C for 24 h. This solution was then added to immobilized antibody beads and incubated for 2 h at room temperature. After washing the beads with PBS (pH 7.2), an aliquot (0.5 μL) was removed for MALDI-TOF analysis.

Acetylation of lysine residues

The monoclonal antibody 5E2.A3 and HIV-p24 were combined, diluted with PBS, and incubated for 2 h at room temperature. The antibody was present in a twofold molar excess relative to HIV-p24 to assure that all HIV-p24 is bound to the antibody. Subsequently, the antibody/antigen complex was digested with endoproteinase Lys-C (enzyme:substrate ratio 1:20) for 12 h. The digest was then split into two equal parts. One part was used directly for the following acetylation reactions. The other part, which was used as a control, was injected into a HPLC system to separate the antigen from the antibody. Fractions of the eluting proteins were collected, lyophilized, redissolved in PBS, and analyzed by MALDI-TOF mass spectrometry. Fractions found to contain fragment 1–158 of HIV-p24 were combined and used for the following acetylation reactions. The identity of this fragment was confirmed by peptide mapping of a tryptic digest of the fragment (data not shown).

The lysine residues in the affinity bound and free fragment (amino acids 1–158) of HIV-p24 (0.1 $\mu\text{g}/\mu\text{L}$) were modified by two successive acetylation reactions using a 10,000- and 100,000-fold molar excess relative to HIV-p24 of acetic and hexadeuteroacetic anhydride, respectively. In the first acetylation reaction, acetic anhydride was added to the solution and the pH was maintained between 6.5 and 7 by dropwise addition of 10% ammonium hydroxide (v/v). The reaction time remained constant (20 min) for all reactions.

Purification and separation from the antibody of the acetylated HIV-p24 fragment were accomplished using HPLC. Fractions (1 mL) were collected, lyophilized, and then redissolved in 50% acetonitrile/0.1% TFA (v/v) for analysis by MALDI-TOF. Fractions found to contain the acetylated HIV-p24 fragment were combined, lyophilized, and redissolved in 50 mM NH_4HCO_3 (pH 7.8). Subsequently, the acetylated fragment 1–158 of HIV-p24 was treated with a 100,000-fold molar excess of hexadeuteroacetic anhydride. The acetylated/trideuteroacetylated fragment 1–158 was purified by HPLC, then the collected fractions were lyophilized and redissolved in 50% acetonitrile/0.1% TFA (v/v). This solution was diluted 1:5 with 50 mM NH_4HCO_3 buffer (pH 7.8), digested with trypsin for 4 h, and the resulting peptides were analyzed by LC-MS. Following tryptic digestion, the peptides were digested further with endoproteinase Glu-C overnight, and the resulting peptides were again analyzed by LC-MS.

Structural images

The structural images of the HIV-p24 protein were created using the coordinates from the solved NMR structure (residues 1–151) (Gitti et al., 1996). The molecular simulations program Insight II (97.0) was used to identify those residues that fell within a 14 Å

radius distance (magenta) from the Pro-1 residue (yellow), and to highlight the lysine residues (turquoise).

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