The Antigenic Determinants on HIV p24 for CD4⁺ T Cell Inhibiting Antibodies as Determined by Limited Proteolysis, Chemical Modification, and Mass Spectrometry

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In the last decade, mass spectrometry has been employed by more and more researchers for identifying the proteins in a macromolecular complex as well as for defining the surfaces of their binding interfaces. This characterization of protein-protein interfaces usually involves at least one of several different methodologies in addition to the actual mass spectrometry. For example, limited proteolysis is often used as a first step in defining regions of a protein that are protected from proteolysis when the protein of interest is part of a macromolecular complex. Other techniques used in conjunction with mass spectrometry for determining regions of a protein involved in protein-protein interactions include chemical modification, such as covalent cross-linking, acetylation of lysines, hydrogen-deuterium exchange, or other forms of modification. In this report, both limited proteolysis and chemical modification were combined with several mass spectrometric techniques in efforts to define the protein surface on the HIV core protein, p24, recognized by two different monoclonal human antibodies that were isolated from HIV⁺ patients. One of these antibodies, 1571, strongly inhibits the CD4⁺ T cell proliferative response to a known epitope (PEVIPMFSALSEGATP), while the other antibody, 241-D, does not inhibit as strongly. The epitopes for both of these antibodies were determined to be discontinuous and localized to the N-terminus of p24. Interestingly, the epitope recognized by the strongly inhibiting antibody, 1571, completely overlaps the T cell epitope PEVIPMFSALSEGATP, while the antibody 241-D binds to a region adjacent to the region of p24 recognized by the antibody 1571. These results suggest that, possibly due to epitope competition, antibodies produced during HIV infection can negatively affect CD4⁺ T cell-mediated immunity against the virus. (J Am Soc Mass Spectrom 2006, 17, 1560–1569)
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Conventional structural biology techniques have been the most powerful avenues for determining the molecular details of protein structure and protein-protein interactions. Understanding protein structure and the protein-protein interactions of the cell at a basic level (i.e., determination of their molecular architectures,

and elucidation of how these molecules interact with one another) is imperative for understanding how the disruption of a single element can result in human disease. Mass spectrometry (MS) is showing tremendous potential for both identification of protein complexes and elucidation of protein structure, in particular for proteins that are not amenable to classical structural techniques. The advantages of MS sensitivity, low sample consumption, and the ability to analyze heterogeneous mixtures can overcome the obstacles that hamper other structural methods. Using MS, in conjunction with limited proteolysis and chemical modification, the structure of a protein or a protein-

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protein complex can be probed by assessing inter-residue distances and changes in solvent accessibility.

One class of protein–protein interaction that can be probed using mass spectrometry is that of antibody–antigen interactions (epitope mapping). Several laboratories have developed mass spectrometric methods for determining the antigenic determinants on a protein using antibody immobilization, proteolytic digestion, differential chemical modification of amino acid residues, and mass spectrometry [1–3]. In the system described here, we examined two antigenic determinants on the human immunodeficiency virus (HIV) core protein p24 and demonstrated the ability to define quaternary protein structures using mass spectrometry.

The HIV/acquired immunodeficiency syndrome (AIDS) global pandemic, of course, is a tremendous burden on human health. Figures from the World Health Organization's December 2005 AIDS epidemic update concluded that ~40 million people are infected with HIV world-wide, with 5 million new cases and 3 million deaths in 2005 alone (ISBN 92 9 173439 X). Hence, intense research has centered on the recognition of HIV by the immune system with much effort directed towards understanding how the virus is able to evade the immune system and ultimately result in AIDS (for review see [4–6]. Understanding these interactions between the immune system and HIV could possibly lead to more effective treatments. Furthermore, elucidation of the molecular details of the immune system's response to HIV infection could also lead to the development of preventative measures such as vaccines (for review see [7, 8]. In fact, much has been learned about the antigenic proteins of HIV and the immune response triggered by the detection of these proteins in the circulation. Antibodies that are capable of neutralizing a broad spectrum of HIV isolates have been documented [9–11], but the presence of these antibodies in HIV⁺ patients is rare, possibly in part because the epitopes these antibodies recognize are fleetingly exposed to the immune system (e.g., a rare conformation of the viral envelope proteins during membrane fusion with the target cell) [11]. The CD4⁺ helper T cell response is another important element of the immune response, in particular for clearing the virus from the infected individual or preventing progression of the infection to AIDS (reviewed in [12]). Unfortunately, strong helper T cell responses are also uncommon in the majority of HIV-infected subjects. The reason behind the lack of strong helper T cell response to HIV is an area of continuing research. Likely, the suppression of the CD4⁺ T cell response is in large part due to the targeted infection of these cells by HIV [13–16], but recent studies have shown that the immune system's own antibodies may also serve to inhibit the CD4⁺ T cell response by binding the antigen from which the epitope displayed by the major histocompatibility complex (MHC) would be derived and somehow block recognition by the T cell [17–22]. Nevertheless, much hope exists in the field for the development of a multiple

antigen vaccine that will induce an immune response that will protect against HIV infection by eliciting a strong T cell response as well as inducing production of broadly neutralizing antibodies.

One HIV protein that is a target for vaccine development is HIV p24 [7, 8, 23–26]. HIV-p24 is the major component of the HIV core and is one of the mature protein products that results from proteolysis of the 55 kD HIV *gag* polyprotein. p24 is of clinical interest because strong CD4⁺ T cell responses against p24 were found to correlate with control of viremia and with lack of progression to AIDS [27]. However, this antigen also elicits high titers of antibodies in the course of HIV infection. Indeed, the presence of antibodies in the serum that recognize p24 was one of the first clinical tests developed to diagnose HIV infection [28, 29]. Antibodies against the conserved regions of this protein also maintain high affinity binding despite the high mutation rate of the virus [30]. Nevertheless, anti-p24 antibodies do not mediate any known antiviral functions. Rather, such high affinity antibodies may interfere with antigen processing and presentation for class II MHC-restricted CD4⁺ T cells. To examine this issue, CD4⁺ T cell proliferation in response to p24 was evaluated in the presence of human monoclonal antibodies (mAbs) specific for this particular antigen. The present study demonstrates that two mAbs from HIV⁺ patients against p24 have distinct inhibitory effects on CD4⁺ T cell proliferation in response to p24. One mAb strongly blocks the T cell proliferation, while the second mAb does not display as strong an inhibitory effect. One possible reason is that the first mAb binds to regions on p24 that are close to or overlap with the T cell epitope, preventing the epitope from being properly presented by the class II MHC to the CD4⁺ T cells. In contrast, the second mAb may recognize a region of p24 that is distinct from the T cell epitope. In efforts to test this hypothesis, the epitopes on p24 that these mAbs recognize were determined.

Experimental

Materials

Recombinant p24 was purchased from Austral Biologicals (San Ramon, CA) or from Protein Science (Meriden, CT). Cyanogen bromide activated sepharose 4B was a product from GE Healthcare Biosciences Corp. (Piscataway, NJ). Modified trypsin was a product of Promega (Madison, WI) and GluC a product of Roche (Indianapolis, IN). BS3 was purchased from Pierce (Rockford, IL) and acetic anhydride, D₆-acetic anhydride, antihuman IgG Fc made in goat, and all other chemicals were from Sigma (St. Louis, MO).

Antibody Production and Purification

Antibody expressing B-cells were isolated from HIV⁺ individuals and immortalized with Epstein-Barr virus,

the resulting cell lines were cultured, and secreted antibodies purified as described by Gorny et al. [31].

T Cell Inhibition Studies

A CD4 T cell clone specific for p24 (AC-25) was examined in this study; the generation and characterization of this clone were reported previously [32]. The clone was human leukocyte antigen-DR-1 (HLA-DR1) restricted as described [32], and its minimal epitope was mapped to PEVIPMFSALSEGATP. T cell proliferative responses to p24 were evaluated in the presence or absence of mAbs in ^3H -thymidine incorporation assays as described previously [18]. Autologous Epstein Barr virus-transformed B cells were used as antigen presenting cells.

Bead Preparation and Antibody Immobilization

Antihuman IgG Fc antibodies were immobilized onto cyanogen bromide activated sepharose 4B beads following essentially the manufacturer's recommended protocol and methods previously established [33]. Briefly, beads were swelled in water and then extensively washed with 1 mM HCl. After equilibration in 50 mM phosphate pH 8.4, 500 mM NaCl, 5 mg of antihuman Fc antibodies per mL of resin were incubated for 1 h at 25 °C with gentle mixing. Unreacted sites on the activated beads were then quenched by the addition of 100 mM Tris pH 8.0. Following another series of washes, 20 μg of either mAbs, 1571, or 241-D, were captured onto the antihuman IgG Fc beads. The resulting antibody complex was then covalently cross-linked by incubating the beads for 1 h with 10 mM BS3. After quenching with 100 mM Tris pH 8.0 and re-equilibration with buffer, the beads could be used immediately or could be stored for up to 1 wk at 4 °C.

Epitope Extraction

Epitope extraction assays were performed essentially as described previously [1–3, 34]. Briefly, p24 was digested to completion with either trypsin or GluC. The resulting digest was then incubated with either the mAb 1571 or the mAb 241-D coated beads. The beads were then washed and analyzed by spotting the resin directly onto the MALDI target to determine if any peptides were retained on the beads.

Epitope Excision

Epitope excision assays were conducted as described by Parker and Tomer [1, 2, 35]. In short, intact p24 was bound to either the mAb 1571 or the mAb 241-D coated beads followed by varying degrees of proteolysis with either trypsin or GluC. After a series of washes to remove unbound peptide fragments, the beads were spotted directly onto MALDI targets and analyzed by mass spectrometry.

Differential Acetylation Studies

Acetylation of primary amines of p24 (11 lysines and the N-terminal amine) were performed either in the absence of any antibody (control set) or in the presence of either the mAb 1571 or the mAb 241-D essentially as described by Hochleitner et al. [36]. Briefly, 2 μg of recombinant p24 in 20 μL of 100 mM phosphate pH 8.0 (4 μM) was treated with 5 mM acetic anhydride for 30 min at room temperature. pH was monitored by pH strip every 5 to 10 min and adjusted upward by the addition of aliquots of 5 M NaOH as needed to maintain a pH >7.0. At the end of the reaction, an equal volume of 1 M Tris pH 8.0 was added to quench any remaining unreacted acetic anhydride. The p24 was then purified by HPLC on an HP 1100 capillary HPLC system removing salts as well as dissociating the p24 from the mAbs. Fractions containing p24, as assayed by MALDI-MS, were pooled and lyophilized. After lyophilization, p24 was resuspended in 20 μL of 100 mM phosphate pH 8.0, 2 M D_6 -acetic anhydride, and allowed to react for 1 h at room temperature. p24 samples were then desalted using C_4 ZipTips from Millipore (Billerica, MA) and the manufacturer's recommended protocol. Acetylated p24 was then digested with trypsin or GluC (pH 4) and MALDI-MS and ESI-MS (see below) were performed on the resulting peptides. Candidate acetylated peptides were identified by first comparing the ions present in each of the acetylated p24 digests that were not present in the control p24 digest. This list of ions was then compared with the theoretical proteolytic digests (up to three missed cleavages) of p24. The ions that corresponded in mass to a predicted proteolytic fragment of p24 plus the mass of one or more acetylations were then analyzed by either MALDI-MS/MS or nano LC-ESI-MS/MS to confirm their identities. Monoisotopic peak heights were used to calculate ratios of H_3/D_3 for the control p24 samples and for the mAb bound p24 samples. By comparing these ratios, the effects that antibody binding has on lysine reactivity and, presumably, lysine surface accessibility were quantitated. Duplicate MS analyses were performed on independent acetylation experiments (at least two) to determine the ratios of acetylation to D_3 -acetylation.

MALDI Mass Spectrometry

Samples (0.5 μL) were spotted onto a 100 sample stainless steel MALDI plate and mixed on target with 0.5 μL of a saturated solution of α -cyano-hydroxycinnamic acid in 45:45:10 (vol:vol:vol) water:ethanol:formic acid. Mass spectrometric analyses were then performed on an Applied Biosystems DE-STR MALDI-TOF mass spectrometer (Foster City, CA) in the positive ion and either linear or reflector modes. The MS spectra were calibrated externally using a mixture of standard peptides. Data were acquired using an accelerating voltage of 25,000 V; grid percentage of 80%; and a delay time of 300 nanoseconds for linear mode analyses and an

accelerating voltage of 20,000 V, grid percentage of 69%, and delay time of 125 nanoseconds for reflector mode analyses. For MS/MS analyses, samples were spotted, 0.3 μ L, onto a 192 sample stainless steel MALDI plate and mixed on target with 0.3 μ L of 33% saturated α -cyano-hydroxycinnamic acid. MS/MS were then performed on an Applied Biosystems 4700 Proteomics Analyzer in the positive ion and reflector modes. The MS/MS calibrated externally using the fragment ions of the angiotensin I M + H ion (m/z 1296.68) with instrument settings of 1000 V for the collision energy and argon as the collision gas with a recharge threshold set at 1.0×10^{-7} torr.

NanoLC ESI Mass Spectrometry

For ESI-MS analyses, samples were introduced into a Q-TOF Ultima mass spectrometer (Waters/Micromass, Altrincham, UK) using flow injection from a pressurized bomb at a flow rate of ~ 300 nL/min. Mass spectrometer settings for MS analyses included a capillary voltage of 3.8 kV, cone voltage of 90 V, collision energy of 10 V, RF setting of 110 V, and a cone gas flow of 50 L/h. NanoLC-ESI-MS/MS analyses were performed using an Agilent 1100 nanoLC system on-line with either a Q-TOF Ultima mass spectrometer or Agilent MDS XCT Ultra ion trap mass spectrometer (Agilent Technologies, Waldbrunn, Germany). Briefly, 8 μ L of 100 nM acetylated p24 digest were loaded onto a PepMap C18 column (75 μ m $\times$ 150 mm, Dionex/LC Packings, The Netherlands) followed by a 15 min wash of 5% acetonitrile, 0.1% formic acid. Peptides were eluted by applying linear gradient from 5% acetonitrile, 0.1% formic acid to 45% acetonitrile, 0.1% formic acid to the column over 45 min. This was followed by a 5 min gradient from 45% acetonitrile, 0.1% formic acid to 95% acetonitrile, 0.1% formic acid and then a 10 min hold at 95% acetonitrile, 0.1% formic acid. MS/MS data were acquired using a data dependent acquisition format. Mass spectrometer settings for MS/MS analyses on the Q-TOF Global Ultima were capillary voltage of 3.8 kV; cone voltage of 90 V; RF setting of 110 V; and a cone gas flow of 50 L/h. Collision energies were applied based on the mass and the charge state of the candidate ions. The Agilent MDS XCT Ultra ion trap mass spectrometer with the chip-cube option was used in the positive ion, ultrascan mode, and included settings of an ionization potential of 1.7 kV and an ICC smart target (number of ions in the trap before scan out) of 100,000 or 500 milliseconds of accumulation. MS/MS analyses were performed using automated switching with a preference for doubly charged ions and a threshold of 5000 counts and a 1.3 V fragmentation amplitude.

Results and Discussion

Because antibodies produced during HIV infection have been shown to have inhibitory effects on the presentation of HIV antigens to MHC class II-restricted

Figure 1. Proliferative responses of p24-specific CD4⁺ T cell clone AC-25 in the presence of human mAbs to p24 or gp120. T cell response was assessed with varying p24 concentrations (4–120 nanomolar), while the mAbs were tested at a constant amount of 67 nanomolar. Recombinant p24 was first incubated with mAbs for 1 h at 37 °C. The p24/mAb mixtures were then added to irradiated APCs. After an overnight incubation, the T cells were added and cultured for 3 d. ³H-thymidine uptake was assessed in the last 18 h of culture. The background response of AC-25 to APCs with no p24 was 408 cpm. Data from one of two independent experiments are shown.

CD4⁺ T cells [18, 19], we investigated the effect of human p24-specific mAbs on CD4⁺ T cell recognition of HIV gag antigen p24. Two anti-p24 mAbs (241-D and 1571) were tested and compared with an irrelevant mAb specific for gp120 (654-D). All of these mAbs were generated from cells of chronically HIV-infected subjects. Of the two anti-p24 mAbs tested, mAb 1571 completely blocked p24-mediated proliferation of the CD4⁺ T cells (Figure 1). The inhibitory effect was observed at each p24 concentration tested. MAb 241-D also reduces p24-mediated proliferation, but only at lower p24 concentrations (Figure 1). In agreement with previously published data [19], anti-gp120 mAb 654-D showed no inhibitory effect (Figure 1). Hence, anti-p24 mAbs 1571 and 241-D displayed distinct effects on CD4⁺ T cell proliferation in response to the p24 antigen.

In efforts to identify the regions of p24 recognized by the CD4⁺ T cell inhibiting mAb 1571 and the less inhibiting mAb 241-D, a mass spectrometry-based approach was employed. Specifically, the purified mAbs were immobilized onto sepharose beads and either the intact p24 antigen or an enzymatic digest of p24 was captured. Both the mAb 1571 and the mAb 241-D coated beads were capable of precipitating the intact p24 antigen as was determined by MALDI-MS analyses (Figure 2a and b). Conversely, under the conditions used in the experiments, neither of the mAb beads was able to precipitate any peptides from the digested p24 antigen (Figure 3a and b). Consistent with these data, both mAbs failed to react with any overlapping synthetic 20-mer peptides spanning the entire p24 protein sequence (data not shown). These results suggest that while both mAbs are able to specifically recognize p24, the epitopes recognized by these antibodies are either discontinuous or linear but conformation dependant (e.g., epitope is part of a tight turn in the protein, but exists as random coil as a digested peptide). The possi-

Figure 2. MALDI-MS showing the singly, doubly, and triply charged states of HIV p24 analyzed directly from 241-D mAb coated beads (a) and 1571 mAb coated beads (b).

bility cannot be excluded, however, that the epitopes are linear and contain proteolytic site(s) such that when they are treated with protease, the epitope is destroyed. To test this possibility, the p24–mAb complex immobilized on beads was subjected to varying degrees of proteolysis with both trypsin and GluC. Interestingly, very few if any peptides are derived from the mAbs themselves. This is believed to be the result of several factors including disulfide bonding and possible inherent resistance imparted by some level of glycosylation, as well as intramolecular cross-links between lysine residues formed during the BS3 treatment. These cross-

links both inhibit tryptic digestion at these lysines as well as prevent release of lysine-containing, cross-linked peptides that are formed by digestion at arginine (trypsin), or aspartate and glutamate (GluC). Extended digestion times (overnight) resulted in no peptides remaining bound to the mAb coated beads (data not shown). These data are interpreted as evidence that both mAbs recognize either linear but conformation dependent or discontinuous epitopes because several examples exist in the literature where a site of proteolysis in a linear epitope is protected from enzymatic digest by antibody binding and is retained on the antibody resin even after long digestion times [1, 2, 36–38]. Furthermore, limited proteolysis (1 h digestion with trypsin at room temperature and a 1:200 enzyme: antigen ratio) followed by MS analyses showed the presence of several proteolytic fragments of p24 remaining bound to the beads coated with the mAb 1571 and one large p24 fragment remaining bound to the mAb 241-D coated beads (Figure 3c and d).

In the case of the mAb 1571, several ions were observed remaining bound to the antibody coated resin (Figure 3c). Singly protonated ions were observed at m/z 7227, 7629, 12470, 15774, and 17080. These ions match closely in mass to the tryptic peptides T4–T6, residues 31–97 ($M_{\text{theor}} = 7224.5$, [M_{theor} are all predicted average masses of singly protonated species] T1–T4, residues 1–70 [residues are numbered from proline 1 of p24, which corresponds to residue 149 of the gag polyprotein; M_{theor} are all calculated average masses of singly protonated species] ($M_{\text{theor}} = 7626.9$), T2–T9, residues 19–132 ($M_{\text{theor}} = 12,466.2$), T1–T11, residues 1–143 ($M_{\text{theor}} = 15771.2$), and T2–T16, residues 19–170 ($M_{\text{theor}} = 17077.6$). Other ions were also observed but are multiply charged species of T1–T11, T2–T9 and T2–T16. All of the peptides observed originate from the N-terminus of the protein with the residues 31–70 represented in all of

Figure 3. Epitope extraction studies indicate no peptides from the completely trypsinized p24 bound to the 1571 mAb (a) or the 241-D mAb (b). MALDI-MS spectrum of epitope excision studies of p24 bound to the 1571 mAb shows multiple peptides from the N-terminus of p24 remain (all with missed cleavages) bound to the mAb coated beads (c). MALDI-MS spectrum of epitope excision studies of p24 bound to the 241-D mAb shows a single peptide from the N-terminus of p24, T5-T12 (residues 71–154), remains bound to the mAb coated beads (d).

(a)

<u>P</u> IVONLOGOM	<u>V</u> HOAISPRTL	<u>N</u> AWVKVVEEK	<u>A</u> FSPEVIPMF
<u>S</u> ALSEGATPO	<u>D</u> LNTMLNTVG	<u>G</u> HQAAMOMLK	<u>E</u> TINEEAAEW
<u>D</u> RVHPVHAGP	<u>I</u> APGOMREPR	<u>G</u> SIDIAGTTST	<u>L</u> OEQIGWMTN
<u>N</u> PPIPVGEIY	<u>K</u> RWIIILGLNK	<u>I</u> VRMYSPTSI	<u>L</u> DIROGPKKEP
<u>F</u> RDYVDRFYK	<u>T</u> LRAEQASQD	<u>V</u> KNWMTETLL	<u>V</u> QANPDCKT
<u>I</u> LKALGPAAT	<u>L</u> EEEMMTACQG	<u>V</u> GGPGHKARV	<u>L</u>

93%

(b)

<u>P</u> IVONLOGOM	<u>V</u> HOAISPRTL	<u>N</u> AWVKVVEEK	<u>A</u> FSPEVIPMF
<u>S</u> ALSEGATPO	<u>D</u> LNTMLNTVG	<u>G</u> HQAAMOMLK	<u>E</u> TINEEAAEW
<u>D</u> RVHPVHAGP	<u>I</u> APGOMREPR	<u>G</u> SIDIAGTTST	<u>L</u> OEQIGWMTN
<u>N</u> PPIPVGEIY	<u>K</u> RWIIILGLNK	<u>I</u> VRMYSPTSI	<u>L</u> DIROGPKKEP
<u>F</u> RDYVDRFYK	<u>T</u> LRAEQASQD	<u>V</u> KNWMTETLL	<u>V</u> QANPDCKT
<u>I</u> LKALGPAAT	<u>L</u> EEEMMTACQG	<u>V</u> GGPGHKARV	<u>L</u>

68%

Figure 4. Sequence of HIV p24 with residues observed by mass spectrometry underlined. (a) Shows sequence observed using a combination of ESI- and MALDI-mass spectrometric analyses of tryptic and GluC digests of unmodified p24 while (b) details sequence coverage of peracetylated p24 using the same biochemical and mass spectrometric techniques.

the peptides, suggesting these residues may constitute the minimal peptide length that is still capable of binding to the mAb 1571. This fragment of p24 comprises all of α -helices 2 and 3, a significant portion of α -helix 4, as well as the loop regions between these α -helices.

Similarly, as seen in Figure 3d, when p24 complexed with the mAb 241-D was digested using conditions that resulted in limited proteolysis, an ion was observed at approximately m/z 9437, closely matching in mass to T5-T12, residues 71–154 ($M_{\text{theor}} = 9435.8$). This peptide is also from the N-terminal domain of p24 and contains the C-terminal portion of α -helix 4, α -helices 5–7 as well as the loops between these helices. T5-T12 was the only peptide observed under the conditions used, suggesting that residues 71–154 is the minimal peptide that maintains high affinity binding with the mAb 241-D.

The results obtained for these limited proteolysis experiments allowed for the rough determination of the regions of p24 recognized by each of these antibodies. Moreover, in addition to trypsin, other proteases including ArgC, chymotrypsin, GluC, and LysC were used for both the epitope excision and epitope extraction experiments. As was seen when trypsin was used, no peptides were retained on the antibodies during epitope extraction, and large fragments from the N-terminus of p24 remained bound during epitope excision assays (data not shown). Due to the multiple fragments observed with in the epitope excision assay with the mAb 1571 and their overlap with the residues identified as remaining bound to the mAb 241-D, as well as the differing phenotype of the two antibodies, a finer characterization of the epitopes was needed to explain the differing biological manifestations of the two antibodies. Therefore, to define the epitopes recog-

nized by each of these antibodies with greater resolution, a differential chemical modification strategy was adopted [36, 37]. Briefly, p24 was acetylated (mass gain of 42 Da) at its lysines when complexed with each of the antibodies, dissociated from the antibodies, and then deuterio-acetylated with D_6 -acetic anhydride (mass gain 45 Da). These acetylated p24 samples were digested with proteolytic enzymes and both MALDI- and ESI-MS were used to identify acetylated peptides based on the presence of ions that were multiples of 42 Da heavier than the predicted peptide masses from theoretical digests of p24 (Figure 4a and b for sequence coverage maps). These ions were further scrutinized by MS/MS analyses. The presence of an acetylated lysine (or the protein's N-terminus) was determined based upon the resulting b- and y-series ions as well as by the presence of the 143.1 Da acetylated lysine immonium ion (Figure 5a). The ratio of acetylation to D_3 -acetylation was determined using peak intensities of the monoisotopic peaks observed in ESI-MS data (Figure 5b). These ratios were then used to assess lysine reactivity in the free and antibody bound p24 and the change in lysine reactivity determined by comparing ratios obtained in the presence of each of the antibodies with the ratio obtained in the absence of any antibody (Table 1). Significance of these values were determined using a t -test with a P value $< .05$.

Figure 5. (a) ESI-MS spectrum of the m/z 1029.6 and m/z 1031.1 ions corresponding to the doubly protonated, acetylated or D_3 -acetylated tryptic fragment T1 of p24 in the absence of antibody. The areas under these peaks were measured and solvent accessibility determined. (b) MALDI-MS/MS spectrum of the singly protonated ion of the N-terminal tryptic peptide of p24 (m/z 2058.8) acetylated on the N-terminal proline with b- and y-series ions labeled.

Table 1. Lysine reactivities of p24

	(p24-1571)/p24	(p24-241D)/p24
N-terminus	0.97 $\pm$ 0.05	0.98 $\pm$ 0.07
25Lys ^a	1.37	1.41
30Lys	0.67 $\pm$ 0.04	1.06 $\pm$ 0.05
70Lys	0.97 $\pm$ 0.09	0.76 $\pm$ 0.11
131Lys	0 $\pm$ 0.02	0.41 $\pm$ 0.02
140Lys	0.73 $\pm$ 0.02	0.58 $\pm$ 0.03
170Lys	0.89 $\pm$ 0.10	1.01 $\pm$ 0.09
182Lys	1.02 $\pm$ 0.22	1.06 $\pm$ 0.19
227Lys ^b	1	1

Lysines, as well as the N-terminus, were modified as described in the methods where the p24 was first modified under mild conditions with acetic anhydride, desalted, and then subjected to conditions that should allow for any remaining unmodified lysines to be deuterio-acetylated by *D*₅-acetic anhydride. The acetylation:*D*₅-acetylation ratio was then calculated for each of the observed lysines for p24 alone, in the presence of the 1571 mAb, or the 241-D mAb. The changes in reactivity in the presence of each of the antibodies were determined by dividing the values. Values significantly greater than 1.0 indicate an increase in reactivity³, suggesting an increase in exposure, significantly less than 1.0 indicate a decrease in reactivity, suggesting protection, and values that do not differ significantly from 1.0 suggest no change in reactivity of the lysine upon antibody binding. At least two independent mass spectrometric measurements were made on at least two independent acetylation reactions and standard deviations are shown.

³[significance determined by t-test with *P* values < 0.05.]

^aSingle measurement.

^bRatios and errors could not be calculated because of the absence of *D*₅-acetylation.

In the instance of the T cell inhibiting mAb 1571, several lysines on p24 were found to have altered reactivity including ³⁰Lys, ¹³¹Lys, and ¹⁴⁰Lys. Upon first inspection, the protection of ¹³¹Lys and ¹⁴⁰Lys does not appear to correlate well with the observation that residues 31–70 may be the minimal binding unit for this mAb. When mapped to the X-ray crystallographic structure (PDB ID 1E6J), however, clearly these lysine residues are in relatively close proximity to residues 31–70 in three-dimensional space (Figure 6a). ³⁰Lys is, of course, immediately N-terminal to the proposed minimal fragment of p24 for mAb 1571 recognition. The protection of ¹³¹Lys by the mAb 1571 binding is not surprising because the side-chain of ¹³¹Lys is oriented towards the proposed minimal binding unit, specifically α -helix 2. In the crystal structure of p24, the ¹³¹Lys side chain is in a position that would suggest the possibility of hydrogen bonds between its amine hydrogens and the backbone carbonyl oxygen of ⁴⁴Ser as well as the carboxylate oxygens of the side chain of ⁴⁵Asp. The protection of ¹⁴⁰Lys by the mAb 1571 is not as readily explained. The side chain of this lysine appears to be directed primarily towards the solvent in the crystal structure of p24 and is not immediately proximal to the putative minimal binding unit of p24, resi-

Figure 6. Ribbon (a) and (b), and space filling (c) and (d) models of p24 (PDB 1E6J) with the regions affected by mAb binding highlighted. The region of p24 (residues 31–70) that remains bound to 1571 mAb coated beads in the epitope excision assay is highlighted in yellow (a) and (c), and the region of p24 (residues 71–154) retained on 241-D mAb coated beads is shown in blue (b) and (d). Lysine residues affected by mAb binding are shown in red (all panels) while the PEVIPMFSALEGATP peptide is shown in green (c) and (d).

dures 31–70. Possibly, even though somewhat removed from the residues involved in the mAb:antigen interface, this lysine side chain is still occluded by antibody binding. Furthermore, while it is postulated that residues 31–70 may be the minimal fragment of p24 recognized by the mAb 1571, it is noteworthy that larger fragments containing ¹³¹Lys and ¹⁴⁰Lys were also in the epitope extraction assays. This indicates that, even though residues 31–70 bind the mAb 1571, an even larger fragment of p24 may make up the complete surface that is recognized by the mAb 1571.

Similarly, differential acetylation studies were performed on p24 in the presence of the less inhibiting mAb 241-D. In these experiments, several lysines of p24 were found to have altered reactivity when p24 was bound to the mAb 241-D. ⁷⁰Lys, ¹³¹Lys, and ¹⁴⁰Lys all exhibited decreased reactivity in the presence of this mAb, correlating well with the epitope excision assay where residues 71–154 were retained by beads coated with the mAb 241-D (Figure 6b). ⁷⁰Lys is immediately N-terminal of the T5-T12 peptide observed in the epitope excision assay while both ¹³¹Lys and ¹⁴⁰Lys are contained within this fragment.

Interestingly, several peptides, both tryptic (excluding those where the cut site was destroyed by acetylation) and those generated by GluC digestion, of p24 that were readily observed when p24 was not acetylated, became difficult to detect by both ESI- and MALDI-MS when the protein was acetylated (Figure 4a and b). In fact, when p24 was digested with trypsin or GluC and analyzed by a combination of MALDI- and LC-ESI-MS, 93% of the sequence of p24, including all lysine residues, was observed when the protein was not acetylated, but when the protein was peracetylated, sequence coverage dropped to 68% with only 8 of 11 lysine residues observed. Possibly, the acetylation of the basic lysine residues decreases the ionization efficiency of the peptides or that the acetylation decreases the polarity sufficiently that the peptides are lost to surfaces during subsequent sample handling due to increased hydrophobicity. For example, peptides in the C-terminal portion of p24 containing ¹⁵⁸Lys, ¹⁹⁹Lys, and ²⁰²Lys, while observed when p24 is not acetylated, are not observed when p24 is acetylated. Presumably, the lack of sequence coverage in these areas upon acetylation is not detrimental because the epitope excision studies demonstrate that only the N-terminal portion of p24 is recognized by the mAbs 1571 and 241-D. Another important example of this effect that does have bearing on the epitope determination of the mAbs 1571 and 241-D is the GluC peptide S1, residues 1–29, which was readily observed when p24 was not acetylated, but only observed once for each mAb when p24 was acetylated. This peptide contains both the N-terminal amine and ²⁵Lys. This peptide demonstrated an increased acetylation to *D*₃-acetylation ratio when p24 was complexed to the antibodies. It is assumed that this increase in acetylation was through ²⁵Lys as the tryptic fragment T1, residues 1–18, and showed no obvious change in the

ratio of acetylation to *D*₃-acetylation at the N-terminal amine. Hence, apparently upon mAb binding, the region of the protein near ²⁵Lys may become more solvent exposed, but the significance of this increased exposure is not known.

The differential acetylation studies provided information about the reactivity of the lysines of p24, and some further refinement of the epitopes on p24 recognized by the T-cell inhibiting mAb 1571 and the less inhibiting mAb 241-D was achieved. The epitope excision experiments yielded large fragments of p24 that comprised large surfaces from multiple faces of the protein. The differential lysine acetylation studies allowed for refinement of these surfaces to a single face for each of the antibodies. In Figure 6a, residues 31–70 of p24 are highlighted. These residues are found both on the “front” of the protein as well as the “back” of the molecule. The lysines that had altered reactivity upon mAb 1571 binding are all on the same side of the protein and narrowed the region of p24 thought to be recognized by mAb 1571.

Similarly, a large fragment of p24, residues 71–154, was found to remain bound to mAb 241-D coated beads. This fragment also comprised multiple faces of p24. The differential lysine acetylation studies enabled further refinement of the mAb 241-D binding surface as well. In Figure 6b the residues 71–154 and the lysines affected by mAb 241-D binding are highlighted. The epitopes that these two mAbs recognize appear to be adjacent with some probable overlap because both mAbs protect ¹³¹Lys and ¹⁴⁰Lys from acetylation.

Interestingly, when the epitope recognized by the p24-specific CD4⁺ T cell line is highlighted along with the epitope mapped for the 1571 mAb (Figure 6c) it is apparent that these epitopes overlap entirely. Because of the direct overlap of the mAb and T cell epitopes, simple competition between the antibody and the T cell for the epitope could be the mechanism by which the 1571 mAb interferes with the T cell proliferative response. On the other hand, the mAb 241-D appears to bind to a surface of p24 that is adjacent to the epitope recognized by the AC-25 CD4⁺ T cell clone used in this study (Figure 6d), possibly resulting in the partial inhibition of the T-cell proliferative response by this antibody. It is more likely, however, that the antibody 241-D functions by an indirect mechanism similar to that as has recently been shown for some HIV gp120-specific antibodies which inhibit the gp120 specific CD4⁺ T cell helper response is by interfering with antigen processing in the antigen-presenting cells [17–20, 22]. In the case of the gp120-specific antibodies, the antibody binding to gp120 confers some resistance to proteolytic enzymes in the endolysosomes such that peptide epitopes are not efficiently released and presented by the antigen-presenting cells [17, 22]. Possibly the antibodies 1571 and 241-D also bind to the N-terminal domain of p24 in such a way that this domain is stabilized and becomes more resistant to endosomal and lysosomal enzymes. The results further imply that

the antibodies maintain high affinity binding to p24 under the acidic conditions found in the lysosomes. Further studies are necessary to test the effects of these antibodies on the presentation of other CD4⁺ T cell epitopes to elucidate each of the multiple possible mechanisms by which these antibodies inhibit the CD4⁺ T cell response against p24.

Conclusions

In this study, mass spectrometry, in conjunction with limited proteolysis and chemical modification, was used to define the regions of a protein involved in a protein–protein interface. Specifically, the protein–protein interfaces between the HIV protein p24 and two different human monoclonal antibodies were determined. The determination of the regions of p24 recognized by each of these antibodies is clinically important because of the potential effects that each of these antibodies may have in vivo. If a relatively conserved region of a p24 protein is determined to be highly antigenic for eliciting responses from CD4⁺ T cells, it could be considered as a lead epitope for vaccine development. However, if this antigenic region elicits competing antibody responses that inhibit the T cell response, then vaccines targeted to this particular region might not be as effective. In the case of T cell epitope PEVIPMFSALSEGATP, this epitope completely overlaps with that recognized by the mAb 1571 and its T cell recognition is strongly blocked by the mAb 1571. Nevertheless, the recognition of this T cell epitope is also affected, albeit weakly, by the mAb 241-D, even though the epitope of this mAb is located in an adjacent nonoverlapping region. The mechanisms by which the mAb 241-D exerts its inhibitory effects remain to be determined, but could be a function of an indirect disruption of antigen processing and presentation as has been observed for other systems. Further characterization is warranted for these two antibodies, as well as for additional antibodies to other regions of p24. These studies should include not only epitope mapping, but also biophysical characterization of the interactions between the antibodies and p24 including binding affinities and thermal stabilities of the complexes.

The mass spectrometry-based approaches described here were an effective method for determining the protein–protein interactions between p24 and the monoclonal antibodies 1571 and 241-D. The limited proteolysis experiments were particularly useful for this antibody–antigen interaction, allowing for quick, albeit low-resolution, determination of the epitopes recognized by each antibody. The chemical modification experiments also showed utility as these experiments allowed for the finer localization of the binding interfaces between the two different antibodies and p24. These differential acetylation experiments, however, do have some limitations. They are labor intensive and require extensive optimization. Moreover, in these studies, it was observed that several peptides of p24, while readily observed in the absence

of acetylation, became difficult to observe when the protein was acetylated. The underlying cause of this effect is not known, but it could be due to the loss of the basic nature of the lysine side chain after acetylation and, hence, loss of ionization efficiency. Another possibility is that a loss of polarity upon acetylation increases the hydrophobicity of these peptides enough that they are lost during subsequent sample handling. Nevertheless, other stable chemical modification techniques, such as protein oxidative surface mapping [39–43], or succinylation of lysines [44, 45] show great promise for mapping protein–protein interactions and will be applied to this as well as other antibody–antigen systems.

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