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Characterization of the Borna Disease Virus Phosphoprotein, p23

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Borna disease virus infection is diagnosed by the presence of serum antibodies reactive with the major viral proteins, p40 and p23. Although p40 and p23 are unrelated in amino acid sequence structure, cross-reactive antibodies are described. Protein fragments and synthetic peptides were analyzed to characterize the specificities of antibodies to p23. Epitope mapping revealed eight continuous epitopes accessible on the surface of a predicted structural model for the monomeric and the disulfide-linked dimeric forms of p23. None of these epitopes was reactive with antibodies to p40. Cross-reactivity with monospecific sera and monoclonal antibodies to p40 was found for one discontinuous epitope located at the amino terminus of p23.

Borna Disease (BD) is an immune-mediated neurologic disease characterized by behavioral disturbances and movement disorders (22, 28, 34, 35). BD virus (BDV), the causative agent, is an enveloped, nonsegmented negative-stranded RNA virus (order *Mononegavirales*) (31). Although natural infection has been confirmed only in horses, sheep, cattle, and cats, additional species, including birds, rodents, and primates, are susceptible to experimental infection (22, 26, 29). Studies of human sera (3, 30, 40) and recent reports of viral nucleic acid in human peripheral blood mononuclear cells suggest that BDV may be implicated in the pathogenesis of human psychiatric diseases (4, 16).

The 8.9-kb viral genome contains five open reading frames (ORFs) (6, 9). In the 5′-to-3′ antigenomic orientation, these ORFs encode proteins of 40 kDa (p40), 23 kDa (p23), 16 kDa (gp18), 57 kDa, and 190 kDa (pol). Proteins p40 and p23 are expressed at high levels in vitro and in vivo and can be detected as early as 24 to 36 h after infection (2). Antibodies directed against p40 and p23 are present in sera and cerebrospinal fluid of naturally and experimentally infected animals (23–25) and are detected before the onset of clinical disease in experimentally infected rats (BD rats) (5). Reports that p40 and p23 copurify in chromatography and immunoprecipitation experiments led to the proposal that they form a complex or have common epitopes (1, 12, 14, 37, 39). Antigen preparations analyzed under nonreducing conditions (NRC) contain a 48-kDa protein (also described as a 60-kDa protein) in addition to p40 and p23. The 48-kDa protein is also found in preparations of purified p23 or recombinant p23 (recp23) studied under NRC and is suggested to represent a dimeric form of p23 (14, 20). Biochemical analysis of p23 has shown evidence for phosphorylation of serine residues but not for other posttranslational modifications (1, 12, 18, 37).

Generation of recp23 and truncated p23 and characterization of the 48-kDa p23 dimer. The full-length cDNA clone of ORF p23 (36) was used as a template in PCRs to generate full-size and truncated cDNA fragments for subcloning into either vector pGEM 7Zf+ (Promega) for in vitro transcription and translation assays or pGEX-KG (11) for expression in *Escherichia coli*. Positions of primers and fragments amplified are indicated in Fig. 1. Primer sequences were as follows: S1, 5′-CATCTAGACATGGCAACGCGACCA-3′; S2, 5′-CATCTAGACATGGCATTGACCCAACCG-3′; S3, 5′-CATCTAGACATGCGCATCGAGGCAGGG-3′; S4, 5′-CATCTAGACATGATGGAGAAGGTGGAC-3′; A1, 5′-GAGCTCTTCGAAGCTTTTTTTTTTTTTT-3′; and A2, 5′-TTTTTTTTTTTTTTTAAGCCGAGATGTCCCC-3′. *Xba*I (TCTAGA) and *Hin*dIII (TTCGAA) restriction sides are underlined. Fragment p23-N1 was amplified with primers S1 and A2 and reamplified for cloning with primers S1 and A1. After expression in *E. coli*, recombinant proteins were purified (33) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular masses were as follows: recp23, 23 kDa; recp23-2, 18 kDa; recp23-3, 9.2 kDa; recp23-4, 5.0 kDa; and recp23-N1, 11.5 kDa (data not shown).

Western immunoblot (WIB) analysis (5) of lysates from infected C6 cells (1, 7) with BD rat serum (15 weeks postinfection [13]) performed under reducing conditions (RC) (100 mM dithiothreitol) or NRC during SDS-PAGE identified proteins of 23, 40, and 48 kDa under NRC but only proteins of 23 and 40 kDa under RC (Fig. 2A). Similarly, WIB analysis of the purified recp23 protein demonstrated both the 23-kDa protein and the 48-kDa protein under NRC but only the 23-kDa protein under RC (Fig. 2A).

To further characterize the nature of the 48-kDa protein, the truncated recp23 fragments (Fig. 1) were analyzed by WIB under RC and NRC. No dimers were observed under RC. Under NRC, dimers were observed with recp23-2 and recp23-3 but not recp23-N1 or recp23-4 (Fig. 2B). The fragments capable of dimerization contain a cysteine at amino acid (aa) position 125 (Fig. 1) (nucleotides [nt] 373 to 375; strain He80-1 [32]).

To test whether disulfide linkage at this residue is essential to dimerization, the cysteine was mutated to serine by PCR. Two fragments that together represented the entire ORF were amplified from ORF p23 cDNA. The point mutation (A to T, nt 373) was contained in a 17-nt region of overlap (nt 367 to 383) between the fragments. Primers were as follows: fragment 1 (nt 1 to 385), T3 promoter (Promega) and 5′-CGGAGTGGTCGCTCCGCTG-3′; fragment 2 (nt 363 to 606), T7 promoter

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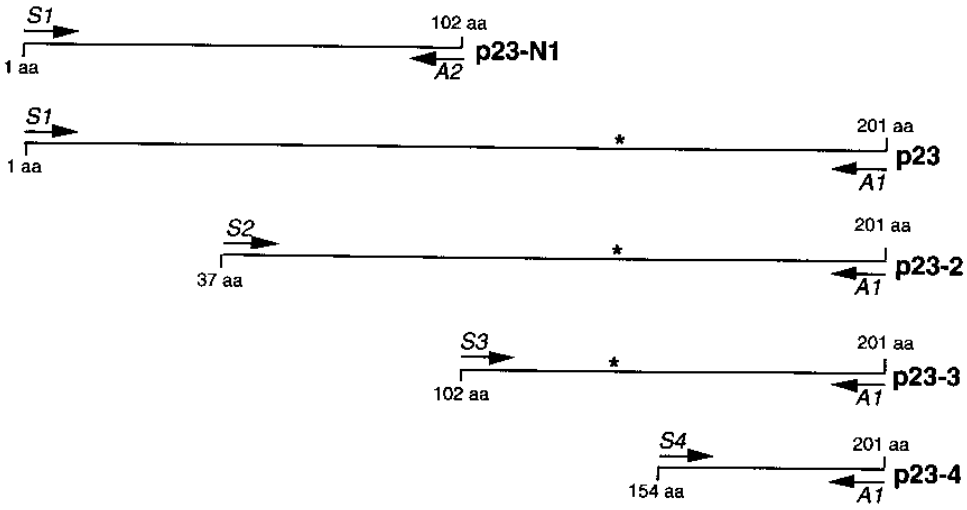

FIG. 1. Plasmid constructs for expression of full-length BDV p23 protein and truncated p23 fragments. Lines indicate the amino acid range of p23 (201 aa) cloned to generate full-length protein (pGEX-p23 and pGEM-p23) and truncated amino-terminal (pGEX-p23-N1 and pGEM-p23-N1) and carboxy-terminal (pGEX-p23-2 to -4 and pGEM-p23-2 to -4) fragments. Positions and orientation of primers used for PCR amplification of the inserts (p23, p23-N1, and p23-2 to -4) are indicated by arrows (see text for sequence of primers). The asterisks indicate the unique cysteine at aa 125.

(Promega) and 5′-AGCTCAGCGGAGCGACCACT-3′. After purification, the two fragments were used in concert as a template to amplify p23(ser) cDNA with primers T3 and T7. The resulting product was cloned into vector pBluescript SK II+ (Stratagene) to generate clone pBDV-p23(ser). Whereas wild-type sequence in pBDV-p23 directed translation of a protein capable of dimerization under NRC, pBDV-p23(ser) directed translation of a protein that did not dimerize under NRC (Fig. 2C).

Identification and mapping of epitopes on p23 detected by sera from infected animals, monospecific sera, and MAbs to p23. Sera from BD rats and infected horses, antibodies to p23 isolated from BD rat sera by binding to recp23 (recp23 eluant) (8, 13), monospecific rabbit sera to recp23 protein (rec23 sera) (5), and monoclonal antibodies (MAbs) to p23 or recp23 were used in WIB analysis, enzyme-linked immunosorbent assays (ELISA), and immunoprecipitation (IP) experiments. In WIB experiments under NRC, all sera and antibodies detected the 48-kDa p23 dimer (data not shown).

The distribution of epitopes on p23 was initially mapped by WIB analysis with fragments recp23-2, recp23-3, recp23-4, and recp23-N1 (Fig. 1). All fragments were detected by rec23 sera (Fig. 2B), sera from BD rats, sera from infected horses, and recp23 eluant (Table 1). recp23 or p23 protein purified from infected MDCK cells was used as an immunogen to obtain murine MAbs x23/8H3, x23/8H5, and x23/9D10 or 23/28A11 and 23/36F1 (37), respectively, from spleen cell fusion clones with the mouse myeloma cell line X63-Ag8.653 (15, 37). The

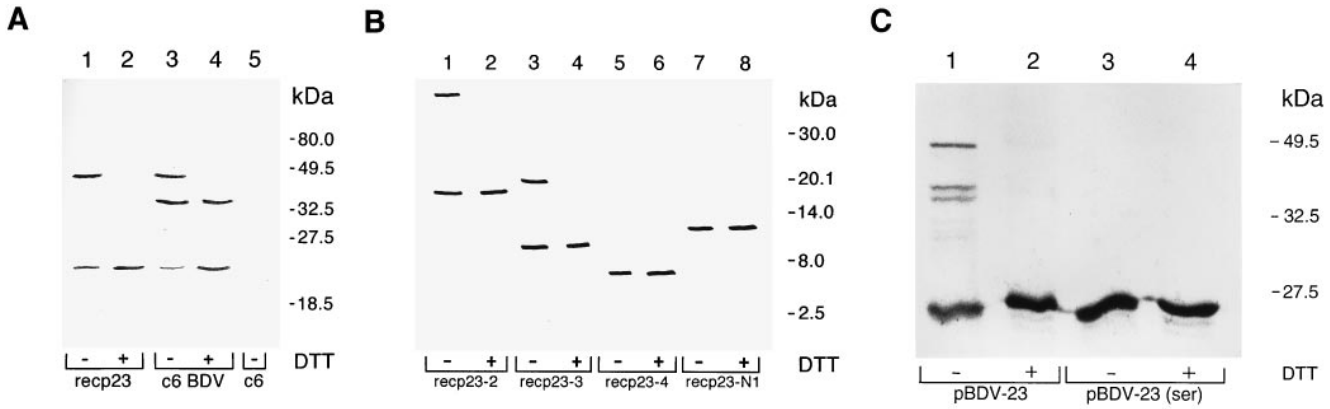

FIG. 2. Characterization of a 48-kDa BDV p23 protein dimer. (A) Detection of a 48-kDa p23 protein complex in cell lysates from infected (lanes 3 and 4) and noninfected (lane 5) C6 cells and preparations of recp23 (lanes 1 and 2; 500 ng each) depending on the use of RC (with dithiothreitol) (+ DTT) or NRC (− DTT) during size fractionation by SDS–10% PAGE. Size-fractionated proteins were transferred to nitrocellulose membranes, antibody labeled with BD rat sera, and visualized by horseradish peroxidase (HRPO)-conjugated second antibody–chloronaphthol immunostaining. (B) Detection of complex formation by p23 fragments recp23-2 (lanes 1 and 2; 500 ng each), recp23-3 (lanes 3 and 4; 1 μg each), recp23-4 (lanes 5 and 6; 2 μg each), and recp23-N1 (lanes 7 and 8; 1 μg each). RC (+ DTT) or NRC (− DTT) were used during size fractionation by SDS–16.5% PAGE. Size-fractionated proteins were transferred to nitrocellulose membranes, antibody labeled with rec23 sera, and visualized by HRPO-conjugated second antibody–chloronaphthol immunostaining. (C) Detection of complex formation by in vitro transcription-translation products generated from pBluescript-p23 (lanes 1 and 2) and pBDV-p23(ser) (lanes 3 and 4). RC (+ DTT) or NRC (− DTT) were used during size fractionation by SDS–10% PAGE. [^{35}S]methionine-labeled transcription-translation products were immunoprecipitated with rec23 sera and protein A-Sepharose prior to analysis by SDS-PAGE and autoradiography. Shorter termination products of p23 (17 and 19 kDa), which also dimerize under NRC, are present in lane 1. Positions of protein molecular mass standards are indicated at the right.

TABLE 1. Characterization of sera and MAbs to BDV p23 and p40

Antibody (no. of samples)	Result by: WIB[a] recp40	recp23	recp23-2	recp23-3	recp23-4	recp23-N1	ELISA[b] (IP[c]) recp40	recp23	recp23-2	recp23-3	recp23-4	recp23-N1
BD rat sera (15 wk postinfection) (4)	+	+	+	+	+	+	+++ (+)	+++ (+)	+++ (+)	++ (+)	++ (+)	+++ (+)
BDV horse sera (4)	+	+	+	+	+	+	++ (+)	++ (+)	++ (+)	+ (+)	+ (+)	++ (+)
rec23 sera	−	+	+	+	+	+	++ (+)	+++ (+)	+++ (+)	++ (+)	++ (+)	+++ (+)
rec40 sera	+	−	−	−	−	−	+++ (+)	++ (+)	++ (+)	− (−)	− (−)	− (−)
recp23 eluant[d] (3)	−	+	+	+	+	+	+ (+)	+++ (+)	+++ (+)	++ (+)	++ (+)	+++ (+)
recp40 eluant[d] (4)	+	−	−	−	−	−	+++ (+)	++ (+)	++ (+)	− (−)	− (−)	− (−)
x23/8H3[e]	−	+	+	−	−	+	+ (+)	+ (+)	+ (+)	− (−)	− (−)	+ (+)
x40/1B5[f]	+	−	−	−	−	−	+ (+)	+ (+)	+ (−)	− (−)	− (−)	− (−)
40/17C1	+	−	−	−	−	−	+ (+)	− (−)	− (−)	− (−)	− (−)	− (−)

[a] +, reactive; −, nonreactive under RC and NRC.
[b] +, titer > 1:100; ++, titer > 1:1,000; +++, titer > 1:5,000. Amount of antigen used per well; 10 ng of recp40, recp23, or recp23-2; 15 ng of recp23-3 or recp23-N1; 20 ng of recp23-4.
[c] +, reactive; −, nonreactive.
[d] Antibodies from BD rat sera eluted from recp40 or recp23.
[e] Identical results were obtained with MAbs x23/8H5, x23/9D10, 23/36F1, and 23/28A11.
[f] Identical results were obtained with MAb 40/15H7.

five MAbs showed identical reaction patterns in WIB experiments, recognizing only full-length recp23, recp23-2, and recp23-N1 (Fig. 3). Similar results were obtained with rec23 sera, sera from infected animals, or MAbs by ELISA with recp23 fragments (Fig. 1) and IP experiments (21) with radiolabeled recp23 fragments generated by in vitro transcription-translation reactions (Table 1). Sera from normal rats, rabbits, and horses and supernatant from native myeloma cells, as well as unrelated MAbs, did not react with p23, recp23, recp23 fragments, or glutathione *S*-transferase in any assay.

The epitopes detected by immunoreactive sera and MAbs were finely mapped in SPOTs tests (Genosys Biotech) using overlapping 8-mer peptides spanning the entire p23 protein synthesized in situ on derivatized membranes. rec23 sera detected eight peptides: e1, PSSLVDSL (aa 5 to 12); e2, NALT QPVDQLLK (aa 38 to 49); e3, DQPTGREQ (aa 61 to 68); e4, VRGTLGDI (aa 94 to 101); e5, TAQRCDHS (aa 121 to 128); e6, METMKLMMEKVD (aa 150 to 161); e7, PMLPSHPA (aa 173 to 180); and e8, TADEWDII (aa 193 to 200). These results

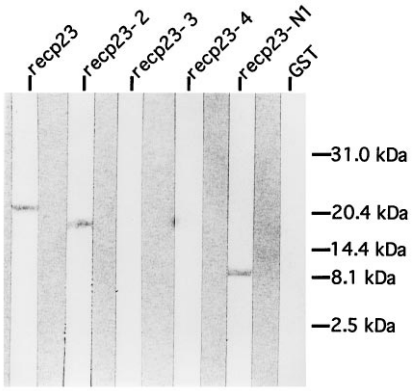

FIG. 3. WIB analysis of truncated p23 fragments with MAb. Full-length recp23; truncated fragments recp23-2, recp23-3, recp23-4, and recp23-N1; and control protein (glutathione *S*-transferase) were transferred to nitrocellulose membrane after size fractionation by SDS–16.5% PAGE under RC. The reactivity of the transferred proteins with MAb 23/36F1 was visualized by horseradish peroxidase-conjugated second antibody–chloronaphthol immunostaining. Positions of protein molecular mass standards are indicated at the right.

were confirmed in ELISA with soluble 14- to 24-aa peptides (500 ng per well) representing epitopes identified in SPOTs tests. All five MAbs detected only peptide e2 in both SPOTs tests and ELISA. Sera from BD rats and infected horses and recp23 eluant detected all eight epitopes (e1 to e8) in ELISA, whereas in SPOTs tests no significant reaction was observed for peptide e7, which had the lowest reactivity among the eight epitopes. No reaction was found in SPOTs tests or ELISA with sera from normal rats, rabbits, or horses; supernatant from native myeloma cells; and MAbs to gp18.

Cross-reactivity of antibodies. Although rec23 sera, recp23 eluant, and MAbs to p23 and recp23 recognized only the p23 protein in WIB experiments, these sera and MAbs also detected recombinant p40 protein (recp40) (5) in ELISA and IP experiments (Table 1). The specificity of the binding to p23 or p40 was analyzed by measuring the dissociation constant (K_D) in an ELISA (10, 19). The K_D values for all MAbs to p23 and recp23 ranged from 10^{-11} to 10^{-9} M when recp23 served as the antigen. In contrast, K_D values ranged from 10^{-6} to 10^{-5} M when recp40 was used as the antigen (data not shown).

To test whether antibodies to p40 were also cross-reactive with p23, monospecific rabbit sera to recp40 protein (rec40 sera) (5), recp40 eluant (8, 13), and three MAbs to p40 (40/15H7, 40/17C1 [37], and x40/1B5, generated by using p40 purified from MDCK cells and recp40, respectively, as the antigen) were tested by WIB, ELISA, and IP experiments. In WIB experiments, sera and MAbs detected only p40 (Table 1) under either RC or NRC. In ELISA and IP experiments, however, rec40 sera, recp40 eluant, and two MAbs detected both p40 and p23. One MAb (40/17C1) showed no cross-reactivity in any assay (Table 1). Similar to MAbs raised against p23 and recp23, which showed markedly lower K_D values for the immunizing antigen (p23) than for the cross-reacting antigen (p40), cross-reactive MAbs 40/15H7 and x40/1B5 had K_D values in the range of 10^{-11} to 10^{-9} M when recp40 was used as the antigen versus K_D values in the range of 10^{-6} to 10^{-5} M when recp23 was used as the antigen (data not shown).

To localize the cross-reactive epitopes on p23, p23 fragments (Fig. 1) were used in ELISA and IP experiments with sera and MAbs to p40. The sera to p40 and the two cross-reactive MAbs reacted with only one p23 fragment, recp23-2

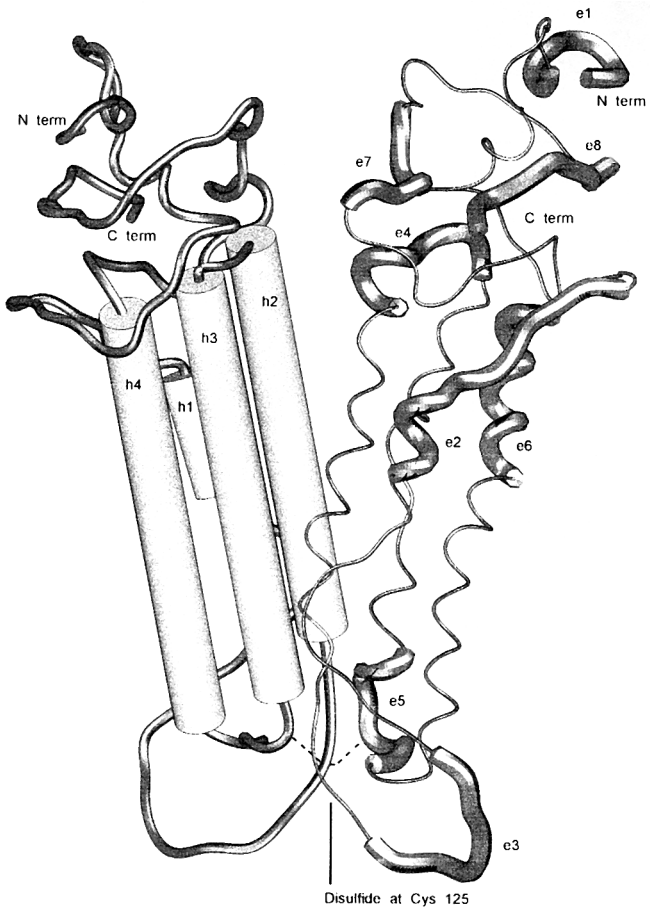

FIG. 4. Three-dimensional model of the BDV p23 protein and its homodimer. The model for the structure of p23 indicates a backbone of four main helices (h1 through h4). The eight continuous B-cell epitopes (e1 through e8) are positioned at surface locations in both the monomer and homodimer of p23. The interrupted line indicates the disulfide bridge linking the monomers. term, terminus.

(Table 1). None of the sera or MAbs to p40 were immunoreactive with p23 peptides in ELISA or SPOTs tests.

Structural model of p23 and its 48-kDa dimer. The p23 amino acid sequence was analyzed by using several secondary-structure algorithms to provide an averaged estimate of secondary structure revealing four main, strongly amphipathic helices (helix 1, aa 42 to 50; 2, aa 78 to 91; 3, aa 106 to 122; and 4, aa 133 to 161). In reviewing other viral proteins, we found similar structures in the coat proteins of cucumber green mottle virus (41) and tobacco mosaic virus (27). By using the helical arrangement of these proteins as a guide for folding order, a three-dimensional model for the p23 monomer was built. Two of these structures are depicted in Fig. 4 in a predicted structural model of the p23 homodimer. The coordinates in protein data bank format are available by anonymous ftp (ftp:// hornet.mmg.uci.edu/pub/lipkin/p23.monomer.pdb or p23.dimer.pdb). A detailed description of the methods used for analyses is available via electronic-mail request to mangalam @uci.edu and via Worldwide Web page at: http://hornet.mmg .uci.edu/~hjm/projects/biocomp/structure.html.

Conclusions. The 48-kDa protein observed in preparations of p23 analyzed in the absence of reducing agents is proposed to represent a disulfide-linked dimeric form of p23 (14, 20). Our model of the p23 monomer places cysteine 125 in an exposed loop between helices 3 and 4, a position suitable for formation of a disulfide linkage (Fig. 4). Consistent with the hypothesis of disulfide linkage at cysteine 125, mutagenesis of this residue to serine abolished dimer formation (Fig. 2C).

The model suggests another feature that may contribute to the three-dimensional organization of the p23 dimer. Although hydrophobic residues located in helix 3 (Ile-88, Phe-108, Val-116, and Ile-119) are present at the surface of monomers, the apposition of these residues in the dimer reduces free energy through enhanced hydrophobic interactions and could orient the subunits as well as stabilize the complex (Fig. 4). Hydrophobic side groups aligned in the methionine-rich portion of helix 4 could also contribute to complex stabilization or interact with other viral or host proteins (38).

Epitope-mapping studies identified eight continuous epitopes along p23; all are present at surface locations in both the monomer and the dimer (Fig. 4). Only one of these epitopes, e2, was recognized by our panel of five MAbs. This finding is consistent with the observation that in rats, antibodies to e2 are present earlier in disease and at higher titers than antibodies to other epitopes (18a).

All five MAbs to p23, rec23 sera, and recp23 eluant cross-reacted with p40 (Table 1). Conversely, two of three MAbs to p40, rec40 sera, and recp40 eluant cross-reacted with p23. There is no similarity between p23 and p40 at the primary amino acid level; thus, it is unlikely that the cross-reactivity reflects similar continuous epitopes. With recp40 and six fragments of p40 in IP experiments, all MAbs to p23 detected only full-length p40 (18b). Both MAbs to p40 that cross-reacted with p23 recognized recp23 and recp23-2 but did not react with any of the synthetic p23 peptides. These findings are compatible with a low-affinity discontinuous epitope being the basis for cross-reactivity. The observation that the affinities of MAbs for the cross-reacting antigens were approximately 4 logarithm units lower than for the immunizing antigens further supports this hypothesis.

The region around epitope e2 to which the cross-reactive MAbs to p23 are directed is highly charged. Similarly, the amino-terminal portion of p40 (aa 1 to 185) contains 51% of all charged amino acids in p40 and includes continuous epitopes recognized by the two MAbs to p40 that cross-react with p23 (40/15H7 and x40/1B5) (18b). The presence of clusters of charged amino acids in proximity to B-cell epitopes at amino-terminal regions of p23 and p40 may be the basis for cross-reactivity. Similarities in antigenic surfaces without amino acid sequence similarities have been demonstrated to be an important mechanism for T-cell epitope molecular mimicry (42).

The presence of charged, discontinuous immunologic determinants on p23 and p40 may have significance for the interpretation of human serologic data. Antibodies reactive with these proteins have been found in sera of patients with neuropsychiatric disorders (3, 30, 40). Recently, BDV nucleic acid sequences were found in peripheral blood mononuclear cells of such patients (4, 16, 17). Intriguingly, there appeared to be no clear concordance between immunoreactivity and the presence of viral sequences: both antibody-positive, nucleic acid-negative and antibody-negative, nucleic acid-positive subjects are reported. It is conceivable that the immunoreactivity for p23 and p40 found for nucleic acid-negative subjects reflects cross-reactivity with the charged, discontinuous determinants on these proteins. Establishing consistent, reliable methods for BDV serology will require the precise definition of these cross-reactive determinants on p23 and p40.

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