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Detailed Mapping of the Antigenicity of the Surface Unit Glycoprotein of Equine Infectious Anemia Virus by Using Synthetic Peptide Strategies

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We describe here a detailed analysis of the antigenic determinants of the surface unit glycoprotein (gp90) of equine infectious anemia virus (EIAV), using a comprehensive panel of synthetic peptides in enzyme-linked immunosorbent assays with immune serum from naturally and experimentally infected horses and with a panel of gp90-specific neutralizing and nonneutralizing monoclonal antibodies. The results of these studies identify immunoreactive segments throughout the conserved and variable domains of gp90 but localize immunodominant (100% reactivity) determinants to the amino and carboxyl termini of the glycoprotein molecule. Analysis of peptide reactivities with longitudinal serum samples taken from experimentally infected ponies revealed that antibody responses to conserved B-cell determinants appeared earlier and at higher titers than do antibodies specific for determinants contained in the variable domain of gp90. These observations suggest an evolution of antibody responses in EIAV-infected ponies that may correspond to the establishment of immunological control of virus replication and disease routinely observed in EIAV infections. In addition, the mapping of monoclonal antibody epitopes to peptides of 9 to 12 amino acids demonstrated that all of the neutralizing epitopes are located in the variable domain of gp90. The arrangement of neutralizing epitopes and critical structural considerations suggest that EIAV gp90 contains a principal neutralizing domain similar to the V3 loop of human immunodeficiency virus type 1. These antigenic analyses provide an important foundation for further analyzing the protective immune response generated during persistent EIAV infections and also provide potential peptide substrates for diagnostic assays and for vaccine strategies.

Equine infectious anemia virus (EIAV) is unique among lentivirus infections in that the infected animal routinely brings virus replication and disease under immunological control. Chronic equine infectious anemia is characterized by a persistent infection in horses that results in recurring cycles of virus replication, plasma viremia, and clinical symptoms (fever, weight loss, hemolytic anemia, edema, leukopenia, thrombocytopenia, etc.) (22, 36). These episodic bouts of viremia and disease appear at irregular intervals separated by weeks or months during the first year of infection. Biochemical and serological studies have shown that the recurrent nature of EIAV can be attributed to the sequential appearance of antigenically distinct viral isolates which arise during the course of a persistent EIAV infection (28, 38, 46, 47, 49, 54). The frequency and severity of these recurring disease episodes decline with time, and the animal typically progresses to an inapparent stage of infection but continues to harbor latent virus (38). The ability to induce productive EIAV expression and clinical disease in these inapparent carriers by stressing or suppressing the immune system indicates that an active immunological management of EIAV virus replication was present prior to intervention (27). Thus, a detailed study of the antigenicity of EIAV antigens and their interaction with the host immune system during the course of a persistent infection can provide important information on the immune status to be achieved by candidate vaccines.

The protective immune response generated during a persistent EIAV infection may be due, at least in part, to the development of a broad spectrum of neutralizing antibodies (37, 53). Detailed studies have demonstrated that the predominant humoral immune response, including virus-neutralizing antibodies, is directed against the envelope surface unit (SU) and transmembrane (TM) glycoproteins of EIAV, gp90 and gp45, respectively (38, 53). DNA sequence analyses of the envelope (*env*) genes of four sequential EIAV isolates from an experimentally infected pony have delineated the clustering of base substitutions into discrete regions of conserved and variable sequences (46). These envelope-specific alterations give rise to antigenically distinct virus strains which can circumvent the current immune status of the host (38, 49, 53, 54). Hence, thorough characterization of the immunogenic components of the evolving surface glycoproteins is critical to the understanding of the relatively rapid dynamics of EIAV replication, disease progression, and eventual control.

Previous investigations have indicated that the predominant antibody response in an infected horse is directed against the peptide backbone of gp90, with only negligible antibody reactivity being oligosaccharide dependent (39). In addition, Payne et al. (48) have broadly defined conserved and variable antigenic segments of EIAV gp90 recognized by equine immune sera in Western immunoblot assays against recombinant fusion proteins. The objectives of this study are to precisely localize critical regions of the EIAV SU glycoprotein which elicit humoral immune responses during a persistent EIAV infection, to map neutralizing and nonneutralizing monoclonal antibody (MAb) epitopes, and to determine the kinetics of humoral immune responses in experi-

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mentally infected ponies. A panel of overlapping peptides which covers the primary amino acid sequence of EIAV gp90 was chemically synthesized and used in immune binding assays against a panel of equine immune sera from natural and experimental EIAV infections, longitudinal immune horse sera, and gp90-specific MAbs. These studies have resulted in a detailed map of key unglycosylated, continuous equine, and monoclonal antigenic determinants that provide useful peptide substrates for diagnostic assays and as peptide immunogens for vaccine trials.

MATERIALS AND METHODS

Equine sera. A previously described standard panel of equine immune sera collected from experimentally infected ponies (E1 to E5) and naturally infected horses (N6 to N20) was used in this study (7, 48). Horses naturally infected in the field with uncharacterized strains of EIAV were selected by positive antibody reactivity in the agar gel immunodiffusion (AGID) assay (Pittman-Moore, Washington Crossing, N.J.). The serum samples from natural infections which were used in this study were acquired 3 to 6 years after the field horses initially tested positive by AGID. Given that these naturally infected horses have reached and maintained long-term immunological management of EIAV replication and disease, the peptide-specific antibody responses seen with sera N6 to N20 reflect the humoral immune status of long-term inapparent EIAV infections. In contrast, experimentally infected ponies were inoculated with characterized EIAV strains derived from the cell-adapted Wyoming prototype virus (31, 52). These serum samples (E1 to E5) were acquired within the first year postinfection. Consequently, the serological reactivity of the experimentally infected horses reflects the immune status obtained relatively early in infection during active, chronic equine infectious anemia. In this way, a direct comparison can be made between the humoral immune status between long-term and early EIAV infections.

Longitudinal equine serum samples were obtained from six ponies that were experimentally infected with either the avirulent, laboratory prototype strain of EIAV or a virulent variant which was derived from the prototype virus (43). Daily temperatures and approximately weekly bleeds were acquired to carefully monitor the clinical course and immune responses as the infection progressed. To monitor the kinetics of production of peptide-specific antibodies and to dissect the humoral immune response early in infection, sera from the weekly bleeds were analyzed in antibody binding assays.

Nonimmune horse sera which was obtained from 10 foals known to be free from EIAV exposure (quarantined farm) and infection (negative for EIAV-specific antibodies by AGID, Western blot, and enzyme-linked immunosorbent assay [ELISA]) were used as negative control sera to detect background immunoglobulin binding in peptide ELISAs.

MAbs. The production and characterization of a limited panel of MAbs to the surface glycoproteins of prototype EIAV have been reported (20, 21). Six of the MAbs (86, 87, 98, 115, 128, and 71) were shown to be EIAV gp90 specific and correspond to the SU epitopes gp90-A, -B, -C, -D, -E, and -F, respectively. Three of the SU MAbs (98, 115, and 128) neutralized prototype infectivity in in vitro neutralization assays. These neutralizing antibodies delineated three distinct epitopes (gp90-C_{NT}, -D_{NT}, and -E_{NT}), all of which displayed variable reactivity with a panel of EIAV isolates.

Prior to use in immune assays, the MAbs were concentrated by ammonium sulfate precipitation and purified by protein A-Sepharose affinity chromatography (SPA-Sepharose; Pharmacia).

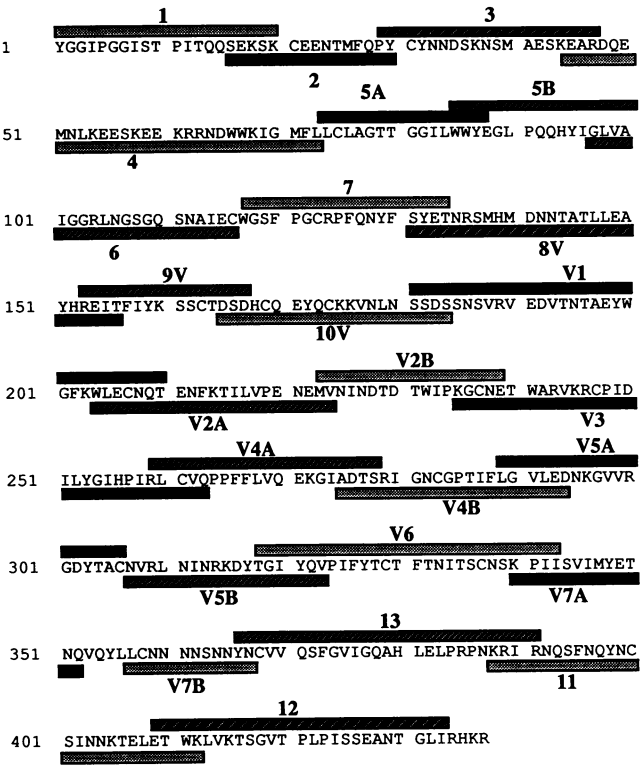

FIG. 1. Linear sequence of EIAV gp90 synthetic peptides. The panel of peptides covering the entire primary amino acid sequence of EIAV gp90 was chemically synthesized according to standard synthetic peptide strategies. The prototype sequence of EIAV gp90 is displayed in a linear fashion, with the SU synthetic peptides delineated by overlapping bars. The shading of the bars is alternated to clarify the overlapping residues contained in individual peptides. Peptides 1 to 7 correspond to the C_N domain; peptides 8V to V7 coincide with the V domain of the prototype EIAV sequence; and peptides 11, 12, and 13 correspond to the C_C domain as defined by Payne et al. (46).

Peptide synthesis. Overlapping peptides covering the primary sequence of prototype EIAV gp90 (46) (Fig. 1) were synthesized either by manual or automated 9-fluorenylmethyloxycarbonyl strategies (5) or by automatic *tert*-butyloxylcarbonyl chemistry (34). Manual syntheses were performed with a DuPont (Boston, Mass.) RAMPS (Rapid Multiple Peptide Synthesizer) system and automated syntheses were performed with a Biosearch (San Rafael, Calif.) SAM II peptide synthesizer according to manufacturer-specified protocols. Coupling and deblocking efficiencies were monitored by the standard ninhydrin assay (9, 25). Peptides were cleaved from their solid resin support and subjected to a series of cold ether extractions, conventional gel filtration chromatography (Sephadex G-25; Sigma Chemical Co., St. Louis, Mo.), and reverse-phase high-pressure liquid chromatography (μBondapak C_{18}; Waters, Milford, Mass.). The final peptide product was characterized by plasma desorption mass spectroscopy (Bio-Ion Nordic AB, Uppsala, Sweden) (9, 24). Only those peptides with the correct theoretical mass were used.

The chemically synthesized peptides shown in Fig. 1 were used as antigens in peptide ELISAs against equine immune

sera and MAbs to detect key B-cell epitopes. The residue numbers for each peptide are relative to the known amino-terminal tyrosine (3). Beginning with tyrosine, the SU amino acid sequence was divided into 11 overlapping segments derived from the conserved amino (C_N) and carboxyl (C_C) domains (peptides 1, 2, 3, 4, 5A, 5B, 6, 7, 13, 11, and 12) and 14 from the central variable (V) region (peptides 8V, 9V, 10V, V1, V2A, V2B, V3A, V3B, V4A, V4B, V5A, V5B, V6, and V7). Additional peptides were subsequently synthesized to localize MAb binding sites to near minimal sequences. These shorter peptides include residues 44 to 52, 50 to 62, and 60 to 72 inclusive of peptide 4; amino acids 183 to 196, 185 to 203, and 195 to 203 comprising peptide V1; residues 264 to 274 corresponding to peptide V4A; and amino acids 300 to 311 overlapping peptides V5A and V5B.

Peptide enzyme-linked ELISA. The standard panels of equine immune sera, normal foal sera (NFS), and MAbs were reacted against each of the SU synthetic peptides in a poly-L-lysine (PLL)-ELISA immunoassay which was developed and optimized to detect bound antibody to peptide antigens (1). Briefly, PLL (Sigma) was bound to the wells of Immulon I (Dynatech, Torrance, Calif.) 96-well microtiter plates and coupled with 1% glutaraldehyde. The synthetic peptide antigens were absorbed to the PLL-coated wells, and nonspecific adherence of antibody was blocked by treatment with a solution of 1 M glycine followed by a 1:1 solution of BLOTTO–1% gelatin (Difco Laboratories, Detroit, Mich.). Serial dilutions of the primary antibody were added in quadruplicate and reacted for 1 h at room temperature followed by a single dilution of peroxidase-conjugated secondary antibody. The calorimetric reaction revealing the presence of bound antibody was initiated by the addition of substrate (*o*-phenylenediamine dihydrochloride; Sigma) and halted by the addition of 2.5 M sulfuric acid. A_{490} measurements were recorded by a MR700 automatic ELISA plate reader (Dynatech).

Positive serological reactivity to the peptides is expressed as a numerical value between 1 and 4 indicating reactivities relative to those of the panel of NFS as described previously (7). The panel of NFS was tested against each peptide in the PLL-ELISA, and the absorbance values were averaged. Immune serum reactivity was defined as 1 if the absorbance was greater than 2.3 times the average absorbance value of the NFS. Numerical values of 2 through 4 reflect absorbances greater than four, six, and eight times the average absorbance of the NFS, respectively. A value of 0 signifies that no antibody binding was detected. Peptides which reacted with 75% or greater of the reference equine sera panel have been designated as immunoreactive, while peptides which reacted with 100% of the equine immune sera panel have been designated as immunodominant.

RESULTS

Localization of MAb binding epitopes. The production and characterization of the six EIAV gp90 MAbs used in this study have been described (20, 21). To localize the corresponding SU MAb epitopes, the gp90-specific MAbs were reacted against the panel of synthetic peptides (Fig. 1) and analyzed for specific antibody binding. The initial MAb reactivity pattern was as follows: MAb 86, specifying epitope gp90-A, reacted with peptide 4 in the C_N domain; MAb 87, defining epitope gp90-B, bound peptide V2B but not V2A or V3A; MAb 98 (epitope gp90-C_{NT}) binding was localized to peptide V4A; both MAbs 115 and 128, corresponding to epitopes gp90-D_{NT} and -E_{NT}, reacted with peptide V1; and the binding of MAb 71, defining epitope gp90-F, was inconclusive. Significantly, in agreement with the previously reported recombinant fusion protein data (48), five of the six MAbs, including the three neutralizing antibodies, were localized to the V region of EIAV gp90.

TABLE 1. EIAV SU MAb epitopes

Epitope	Residue no.[a]	Amino acid sequence[b]	% Reactivity[c]
gp90-A	44-52	KEARDQEMN	30
gp90-B	225-234	NINDTDTWIP	45
gp90-C_{NT}	264-274	PPFFLVQEKGI	45
gp90-D_{NT}	195-203	NTAEYWGFK	15
gp90-E_{NT}	185-194	SNSVRVEDVT	40
gp90-F	?[d]	?	ND[e]

[a] Starting from the mature gp90 amino-terminal tyrosine as residue 1 (3).
[b] EIAV prototype sequence as determined by Rushlow et al. (52)
[c] Serological reactivity of the MAb determinants against the standard panel of equine immune sera, E1 to E5 and N6 to N20.
[d] MAb 71 corresponding to epitope gp90-F was unreactive with the panel of synthetic peptide antigens in ELISAs. This epitope appears to be conformational.
[e] ND, not determined.

To further localize the mouse epitopes to near minimal sequences, smaller overlapping peptides covering the sequences of each of the previously reactive peptides were synthesized and reacted against the MAbs in PLL-ELISAs. As shown in Table 1, antibody reactivity delineated five of the six gp90 epitopes to 9 to 12 amino acid residues. It is noteworthy that neutralizing MAbs 115 and 128, which correspond to epitopes gp90-D_{NT} and -C_{NT}, mapped to contiguous but nonoverlapping peptide sequences, while the third neutralizing MAb epitope (gp90-C_{NT}), defined by MAb 98, mapped 57 amino acids downstream. Thus, the three neutralizing MAb epitopes have been localized to two discrete, closely positioned segments in the V domain of the SU glycoprotein. MAb 71, which specifies epitope gp90-F, failed to react with any of the peptide substrates. The inconclusive reactivity of MAb 71 against synthetic peptide antigens and positive binding activity with intact gp90 suggest that epitope gp90-F may be conformation dependent. Previous investigations have broadly associated epitope gp90-F with the V domain (21, 48). The locations of the MAb epitopes defined in these studies are summarized in Fig. 2.

To determine whether the MAb-defined peptide epitopes are also immunogenic in horses during persistent EIAV infections, the panel of immune equine sera was reacted against each of the peptides corresponding to MAb determinants. Shown in Table 1 are the equine serological reactivities against the shorter synthetic peptides representing the MAb epitopes. The MAb peptides displayed positive reactivity with 15 to 45% of the reference equine sera. Thus, it appears that each of the peptide sequences corresponding to MAb epitopes can induce antibodies in virus-infected horses. Although none of the MAb epitope peptides reacted with the majority of the equine immune sera, four of the MAb epitope sequences overlap larger, highly reactive peptide segments (see below). The noted reduction in positive equine serological reactivity to the MAb determinants compared with the reactivity of the larger corresponding peptides suggests that the equine polyclonal immune sera bind multiple epitopes in the larger peptide fragments.

Reactivity of equine immune sera against peptides corresponding to the C_N and C_C domains of EIAV gp90. Synthetic peptides which correspond to the conserved sequences of

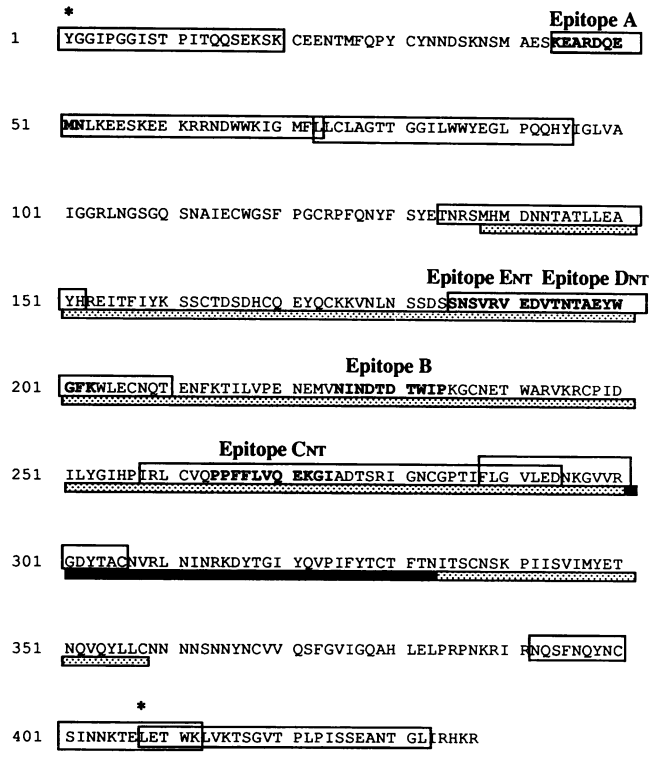

FIG. 2. Linear representation of EIAV SU epitopes. Standard panels of MAbs and immune equine sera were reacted in ELISAs against the panel of EIAV SU synthetic peptides. MAb and equine humoral epitopes are portrayed along the linear amino acid sequence of EIAV gp90. The residues corresponding to the SU MAb epitopes A to E are indicated in boldface, with epitopes C, D, and E being neutralizing (NT). Boxed sequences delineate immunoreactive peptides which are recognized by 75% or more of the equine immune sera. The boxed sequences marked with asterisks depict immunodominant peptides that are immunoreactive with 100% of the EIAV-positive sera tested to date. The underlined sequence coincides with the V domain as defined by Payne et al. (46). The darkened underline offsets a V_H segment within the V domain.

EIAV gp90 were reacted against the equine immune sera to dissect conserved fragments of the SU molecule that are immunogenic during a persistent EIAV infection, that delineate conserved immunodominant domains, and that reveal potential diagnostic reagents. The relative reactivities obtained against the conserved SU peptides from both the C_N and C_C domains of EIAV gp90 are shown in Table 2. The locations of the immunoreactive and immunodominant peptide determinants identified in this study are summarized in Fig. 2. Three immunogenic B-cell determinants, as defined by 75% or greater positive reactivity with immune horse sera, were identified in the C_N domain. The amino-terminal peptide (peptide 1; residues 1 to 20) of mature gp90 reacted with relatively weak intensity with 100% of the equine immune sera tested. Immune horse serum reactivity decreased to 60% with peptide 2 (residues 16 to 30), delineating the immunodominant (100% positive serological activity) amino-terminal equine epitope to the first 20 amino acids of mature gp90. As indicated in Table 2, peptide 4 (residues 44 to 70) includes the second immunogenic B-cell determinant in the C_N domain, displaying 80% positive serological reactivity with the panel of immune horse sera and a stronger relative intensity with experimentally infected sera. Peptide

TABLE 2. PLL-ELISA results from the conserved segments[a] of EIAV gp90

Horse serum[b]	Relative binding[c] to peptide:										
	N terminal								C terminal		
	1	2	3	4	5A	5B	6	7	13	11	12
E1	1	3	2	3	2	1	1	1	1	4	4
E2	1	2	0	3	2	1	0	0	0	4	4
E3	2	1	0	1	1	1	0	2	0	3	4
E4	1	0	0	1	0	0	1	0	1	0	4
E5	2	3	0	3	2	2	3	1	1	4	4
N6	1	0	0	0	2	3	0	0	0	1	4
N7	2	4	0	1	0	1	1	1	1	3	4
N8	3	1	1	2	1	2	2	1	1	2	4
N9	3	0	0	1	1	1	1	0	1	3	4
N10	2	0	0	0	1	1	0	0	0	0	4
N11	1	1	0	1	0	0	2	1	1	0	4
N12	1	1	0	1	1	1	3	0	1	1	4
N13	2	0	0	0	1	1	1	0	1	1	4
N14	2	1	0	1	1	1	1	1	1	2	4
N15	4	2	0	1	2	2	1	0	2	2	4
N16	1	0	0	1	1	1	0	1	0	3	4
N17	1	1	0	1	1	1	1	0	0	3	3
N18	1	0	0	0	0	0	0	0	0	1	2
M19	1	0	0	1	1	1	1	0	0	1	4
N20	2	1	0	1	1	1	1	1	1	2	4
% Positive	100	60	10	80	80	85	70	45	60	85	100

[a] As defined by Payne et al. (46).

[b] Standard panel of equine immune sera. E1 to E5 are from experimentally infected ponies; N6 to N20 are from natural infections by uncharacterized EIAV in field horses.

[c] 0, no antibody binding to peptide detected; 1, absorbance greater than 2.3 times the average absorbance of NFP; 2, absorbance greater than 4 times the average absorbance of NFP; 3, absorbance greater than 6 times the average absorbance of NFP; 4, absorbance greater than 8 times the average absorbance of NFP.

4C (amino acids 44 to 52), corresponding to MAb epitope gp90-A, weakly reacted with only 15% of the equine immune sera (Table 1), indicating that the immunogenic segment of peptide 4 is located between residues 53 and 70. The final immunogenic region in the C_N domain consists of the two overlapping peptides, 5A and 5B (residues 73 to 98), which exhibited 80 and 85% positive serological reactivity, respectively (Table 2).

Other peptides included in the C_N domain are peptide 3 (residues 29 to 47), peptide 6 (residues 97 to 116), and peptide 7 (amino acids 117 to 134). Peptide 3, which contains the highly conserved amino-terminal cysteine (residue 31) and a potential N-linked glycosylation site (amino acids 34 to 36), reacted with only 10% of the reference equine sera at relatively low intensity. This low serological reactivity could reflect the sequestering of the peptide sequence in native gp90 by oligosaccharides or, perhaps, indicate a conformational epitope dependent upon the formation of disulfide linkages involving the highly conserved cysteine. In contrast, peptide 6 exhibited 70% serological reactivity with the equine immune sera tested, while relatively weak reactivity with 45% of the equine immune sera was seen with peptide 7.

Three synthetic peptides comprise the C_C domain and display a gradient of serological reactivity. The carboxyl-terminal residues of EIAV gp90 (peptide 12; amino acids 409 to 434) reacted with 100% of the reference equine immune sera at a strong relative intensity. Both the frequency and the intensity of the carboxyl-terminal reactivity decreased as the

TABLE 3. PLL-ELISA results with peptides from the variable segment[a] of EIAV gp90

Horse serum[b]	Relative binding[c] to peptide:													
	8V	9V	10V	V1	V2A	V2B	V3	V4A	V4B	V5A	V5B	V6	V7A	V7B
E1	4	0	0	4	0	0	0	0	0	0	1	0	0	0
E2	2	0	0	4	1	0	0	0	0	0	0	0	0	0
E3	3	0	0	4	0	0	0	0	0	0	0	0	0	0
E4	2	0	0	1	0	0	0	1	1	0	1	0	0	1
E5	4	0	0	4	0	1	1	1	1	1	2	0	0	2
N6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N7	4	0	1	1	1	1	1	1	1	0	4	0	0	1
N8	3	0	1	1	1	2	3	2	2	1	2	2	0	2
N9	4	0	0	0	1	1	2	2	2	1	1	0	0	4
N10	0	0	0	0	0	0	0	1	1	0	0	0	0	0
N11	1	0	0	3	1	0	1	1	1	1	1	1	0	1
N12	2	0	0	1	1	1	1	2	2	1	1	1	1	3
N13	0	0	0	0	0	0	3	1	1	1	0	1	0	2
N14	1	0	0	2	1	1	2	2	2	1	1	2	0	2
N15	1	0	0	2	1	1	3	3	2	1	3	1	0	2
N16	4	0	0	1	0	0	2	1	1	2	4	1	0	0
N17	1	0	0	1	1	2	3	1	1	1	1	2	0	2
N18	0	0	0	1	0	0	1	1	2	0	1	1	0	1
N19	1	0	0	0	0	0	4	1	2	1	1	0	0	0
N20	1	0	0	3	0	2	4	2	2	1	2	1	0	2
% Positive	80	0	10	75	45	45	70	80	80	60	80	50	5	65

[a] As defined by Payne et al. (46).

[b] Standard panel of equine immune sera. E1 to E5 are from experimentally infected ponies; N6 to N20 are from natural infections by uncharacterized EIAV in field horses.

[c] 0, no antibody binding to peptide detected; 1, absorbance greater than 2.3 times the average absorbance of NFP; 2, absorbance greater than 4 times the average absorbance of NFP; 3, absorbance greater than 6 times the average absorbance of NFP; 4, absorbance greater than 8 times the average absorbance of NFP.

peptide segments moved further upstream into the core of the gp90 molecule (Table 2). Peptide 11 (residues 388 to 412), located immediately upstream of the immunodominant peptide 12 (Fig. 1), reacted at a relatively moderate intensity with 85% of the equine immune sera analyzed, and the adjacent peptide (peptide 13; residues 367 to 392) displayed 60% serological reactivity of relatively weak intensity. Thus, the immunodominant, C-terminal equine determinant is localized to the carboxyl 20 amino acids of gp90 and displays the strongest relative reactivity of all peptides tested to date, including peptides derived from the TM protein of EIAV (7).

Reactivity of equine immune sera against peptides corresponding to the V domain of EIAV gp90. Synthetic peptides derived from the V region of EIAV gp90 in general exhibited an overall lower reactivity with the reference equine sera than was observed with those peptides corresponding to conserved sequences of gp90 (Table 3). For example, only peptides 8V and V1 displayed a significant number of level 4 reactivities with the equine serum panel, and these highly reactive sera were typically from the experimentally infected ponies. This higher level of reactivity in sera E1 to E5 probably reflects a type-specific antibody response against laboratory strains of virus closely related to the prototype EIAV sequence used as a basis for peptide synthesis.

Peptide 8V (residues 131 to 156) exhibited 80% serological reactivity with the equine immune sera and 100% reactivity of relatively high intensity with the experimentally infected sera. The next overlapping peptide sequence, peptide 9V (residues 152 to 167), did not react with any of the sera tested, and only 10% of the sera bound the adjacent peptide (peptide 10V, amino acids 155 to 184). Taken together, these data suggest the presence of an immunogenic segment in the V domain between residues 131 and 151. Extensive amino acid sequence variation in the region of EIAV gp90 corresponding to peptides 9V and 10V could preclude notable cross-reactivity of these peptide segments, or possibly this segment of gp90 is not exposed in the native protein structure.

Peptide V1 (residues 181 to 210) is included in a region of gp90 which appears to be functionally equivalent to the primary neutralizing domain of human immunodeficiency virus type 1 (HIV-1) that is contained in the V3 loop of gp120 (10, 23, 33, 44). This variable segment of EIAV gp90 has been designated the principal neutralizing domain (PND) (36). The peptide V1 sequence contains two of the three defined neutralizing MAb epitopes, reacts with 75% of the immune horse sera tested, and like peptide 8V, displays a stronger intensity with sera from experimentally infected ponies (E1 to E5) than with the sera from field-infected horses (N6 to N20). To examine the serological reactivity of the individual neutralizing MAb determinants contained in peptide V1 (gp90-D_{NT} and gp90-E_{NT}), the panel of equine immune sera was reacted against the peptides corresponding to the MAb epitopes. The serological reactivity of the equine immune sera was significantly reduced at 15 and 40% (Table 1), respectively, from the 75% positive reactivity observed with the larger V1 peptide. Evidently both MAb binding sequences are critical components of this immunoreactive segment recognized by the horse.

Peptides V4A (residues 259 to 279), V4B (amino acids 275 to 294), and V5B (residues 307 to 323) each reacted with 75% or greater of the panel of immune horse sera, indicating the presence of multiple immunogenic fragments in the V domain. The neutralizing MAb epitope gp90-C_{NT} was mapped to a portion of peptide V4A (amino acids 264 to 274) which reacted with 45% of the equine immune sera tested (Table 1). The 70% positive serological reactivity directed against peptide V3 (amino acids 235 to 263) was predominantly

TABLE 4. PLL-ELISA results of longitudinal equine immune sera with the EIAV SU peptides

Days postinfection	Relative binding to peptide:															
	C_N domain								V domain[a]					C_C domain		
	1	2	3	4	5A	5B	6	7	8V	V1	V4A	V4B	V5B	13	11	12
With avirulent prototype EIAV[b]																
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	4
24	0	0	0	4	0	0	1	0	0	1	0	0	0	0	0	4
42	0	0	0	2	0	0	0	0	0	3	0	0	0	0	0	4
49	1	0	0	3	0	0	0	0	1	3	0	0	0	0	0	4
55	1	0	0	4	0	0	0	0	1	2	0	0	0	0	0	4
62	1	0	0	4	0	0	0	0	1	2	0	0	0	0	0	4
69	3	0	0	4	0	0	0	0	1	2	0	0	0	0	0	4
77	4	2	0	4	0	0	0	0	2	4	0	0	0	1	0	4
84	4	2	0	4	0	0	0	0	2	3	0	0	0	2	0	4
119	4	3	0	4	0	0	0	0	2	3	0	0	0	2	0	4
With virulent strain of EIAV[c]																
8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	4	0	3	1	0	0	0	0	0	2
24	0	0	0	2	0	4	2	0	4	3	0	0	0	0	4	4
28	0	0	0	4	0	4	3	0	4	3	0	0	0	0	4	4
35	0	0	0	4	0	4	2	0	4	2	0	0	0	0	4	4
42	0	0	0	4	0	4	1	0	4	1	0	0	0	0	4	4
45	0	0	0	4	0	4	4	0	4	1	0	0	0	0	4	4
49	1	0	0	4	0	4	2	0	2	3	0	0	0	0	4	4
55	2	0	0	4	0	4	1	0	2	3	0	0	0	0	3	4
65	1	0	0	4	0	4	2	0	2	3	0	0	0	0	3	4
77	1	0	0	4	0	4	1	0	1	4	0	0	0	0	2	4
84	1	0	0	4	0	4	1	0	1	4	0	0	0	0	2	4
91	1	0	0	4	0	4	1	0	1	4	0	0	0	0	2	4
105	1	0	0	4	0	4	1	0	0	4	0	0	0	0	1	4
119	1	0	0	4	0	4	1	0	0	4	0	0	0	0	1	4
140	0	0	0	4	0	4	1	0	0	4	0	0	0	0	1	4
153	0	0	0	4	0	4	1	0	1	4	0	0	0	0	0	4

[a] Selected synthetic peptides from the V region which were immunoreactive with the standard panel of equine immune sera. Peptides V7A and V7B reacted intermittently at a relative intensity of 1; all other peptides from the V domain were nonreactive with the longitudinal immune sera and are not shown.
[b] Longitudinal immune sera from a pony infected with the avirulent, prototype strain of EIAV.
[c] Longitudinal immune sera from a pony infected with a virulent variant of prototype EIAV.

found in long-term naturally infected sera (N6 to N20), with very little reactivity seen with sera from experimentally infected ponies (E1 to E5). Only a single horse reacted at relatively low intensity with peptide V7A (amino acids 339 to 352), while 65% of the standard equine serum panel displayed moderate reactivity against peptide V7B (residues 357 to 367). The remaining peptides derived from the V domain, V2A (residues 204 to 224), V2B (amino acids 223 to 239), V5A (amino acids 289 to 306), and V6 (residues 318 to 343), displayed a weak, scattered reactivity pattern (Table 3).

Reactivity of longitudinal equine immune sera. Six ponies were experimentally infected with either the avirulent prototype strain of EIAV or a virulent variant of the prototype virus. Approximately weekly bleeds were acquired to carefully monitor the humoral immune response as the infection progressed. Regardless of the virus inoculum, all of the EIAV experimentally infected ponies seroconverted (positive by AGID) between 17 and 21 days postinoculation (dpi), and virus was recovered by 10 to 17 dpi. Hence, a uniform immune response was observed under the experimental conditions used, such that a direct comparison of envelope glycoprotein-specific antibodies can be made between the virulent and avirulent infections. To closely evaluate the kinetics of the humoral immune response directed against the conserved and variable components of EIAV gp90, the longitudinal immune sera panels were reacted against the complete panel of SU synthetic peptides.

Sera from the weekly bleeds of the three ponies that were infected with the avirulent prototype EIAV were examined from 8 dpi through 119 to 146 dpi for immune reactivity against each of the SU peptides composing the panel of peptides. Table 4 summarizes the serological data from a representative prototype EIAV-infected pony. Significantly, no serological reactivity was observed over the sampling period against the variable peptides 9V, 10V, V2A, V2B, V3, V4A, V4B, V5A, V5B, and V6 or the conserved peptides 3, 5A, 5B, 7, and 13, while strong relative reactivity was observed with the conserved peptides 4 and 12 (Table 4). The strong relative reactivity demonstrated with peptides 4 and 12 and the absence of immune reactivity noted with peptides 3, 9V, and 10V concur with the results obtained with the standard panel of immune sera (Tables 2 and 3). In contrast, peptides V4A, V4B, and V5B, which were relatively immunoreactive with the standard immune serum panel, displayed no antibody binding activity with any of the early sera from experimentally infected ponies. These data indicate that these variable peptides may be immunoreactive

only after an extended chronic EIAV infection. Peptides 6, V1, and 11 were nonreactive in at least one of the prototype-infected animals, and peptides 1 and 8V displayed moderately positive reactivities between 24 and 49 dpi. Peptide 2 moderately reacted with sera after 69 dpi, and variable reactivity was obtained against peptide 13. The remainder of the peptides were generally nonreactive over the time tested.

Longitudinal sera from the three ponies infected with the virulent EIAV strain tested with an overall higher reactivity that was sustained over time (153 dpi). Table 4 shows the serological data from a representative pony infected with a virulent EIAV variant, which indicate an evolution of peptide-specific humoral immune responses. Peptides 4, 8V, V1, 11, and 12 demonstrated positive reactivity by 24 dpi and remained strongly positive, with a relative reactivity of 4 in all three of the ponies infected with the virulent EIAV. A single pony displayed a sustained relative reactivity of 4 against peptide 5B. Similar to the avirulent prototype-infected animals, variable peptides 9V, 10V, V2A, V2B, V3, V4B, V5A, V5B, and V6 were nonreactive over the time span analyzed (Table 4). Peptide V4A reacted intermittently with two of the three pony sera. Serological reactivities against peptides 1 and 6 were moderate and somewhat variable in intensity, while the remainder of the SU peptides were essentially nonreactive.

These data demonstrate that the conserved peptides 4 and 12 were detected early in sera from both prototype and variant EIAV-infected animals and that these peptides remained positive, with a high relative absorbance value in all six of the ponies tested. In general, peptides coinciding with variable sequences were remarkably unreactive, suggesting that animals must be infected long term and be exposed to several distinct viral isolates before they develop antibodies that bind variable sequences. This is significant given that all three neutralizing MAb epitopes have been isolated to the V domain. Two exceptions were the variable peptides 8V and V1, which displayed significant serological reactivity against the longitudinal equine immune sera (Table 4).

To evaluate further the evolution of peptide-specific humoral immune responses, sera were taken 63 days and 14 months postinfection from two prototype-infected horses and tested against the panel of SU peptides (data not shown). The 63-dpi samples were selected since early immune responses appear to be stable by 62 to 65 dpi (Table 4), and the 14-month samples were chosen to reflect the immune status after 1 year postinfection. Notably, peptides V2A, V2B, V3, V5B, V6, and 11, which were nonreactive with the longitudinal prototype-infected sera (Table 4), were reactive with both of the 14-month serum samples. In agreement with the serological reactivity of the longitudinal sera, peptides 3, 6, and 7 were nonreactive, while peptides 2, 4, 8V, and 12 were highly reactive. These results suggest that the previously characterized broadening of humoral immune responses during persistent EIAV infections (53) may correlate to the evolution of antibody responses to the variable sequences of EIAV gp90.

DISCUSSION

Synthetic peptides have been used to map antigenic sites in a number of viral systems, including extensive analyses of HIV glycoproteins (17, 26, 40, 42, 58). Although peptide antigens are designed to identify specific linear sites which are immunogenic in the native protein antigen, the level of seroreactivity with any peptide is influenced by several parameters. The particular conformational properties of the peptide antigen may vary from the native protein structure and thus reduce antibody binding. In addition, the presence of major histocompatibility complex polymorphism in an outbred population influences the recognition of a particular peptide antigen by the immune system (4). Results of several studies have indicated that the recognition of peptide antigens by sera from HIV-1-infected individuals vary among the infected population and during the course of infection in a particular person (29, 35, 40). In certain cases, near 100% reactivity with a peptide antigen has been observed, as in the case of the immunodominant TM peptide (15, 41, 57) and the carboxyl terminus of the SU glycoprotein of HIV-1 (45). In general, 50% and greater positive serological reactivity with a reference panel of immune sera against HIV-1 envelope antigens has been considered to represent a significant B-cell epitope (29, 40, 42, 58). However, other studies have defined B-cell epitopes with considerably lower reactivities (16, 56).

In this study, we used a systematic, empirical approach utilizing a panel of synthetic peptides covering the complete sequence of prototype EIAV gp90 to localize distinct B-cell epitopes reactive with both experimentally and naturally infected equine immune sera and to map neutralizing MAb epitopes. The most significant results of this study are the identification of highly reactive B-cell determinants and a potential diagnostic peptide antigen, the elucidation of key differences in early and late humoral immune responses of EIAV-infected horses, the localization of neutralizing sites and a PND, and the evaluation of the kinetics of antibody responses to variable and conserved antigenic determinants. A limitation of this study, however, is that the use of synthetic peptides cannot identify discontinuous conformation-dependent epitopes that may in fact represent important antigenic determinants of EIAV gp90.

Serological reactivities of a panel of equine immune sera have delineated antigenic determinants (75% and greater positive reactivity) throughout the sequence of EIAV gp90 and two immunodominant epitopes (recognized by 100% of the equine immune sera tested) to the amino and carboxyl termini. As summarized in Fig. 2, two immunoreactive sequences (residues 44 to 70 and 73 to 98), an immunodominant peptide (amino acids 1 to 20), and the conserved MAb epitope (gp90-A) have been localized to the C_N domain. A distinct peptide sequence (residues 55 to 65) in the first conserved domain (C1) of HIV-1 gp120 has been reported to be immunoreactive with AIDS patient sera (16). Thus, it appears that the conserved, amino-terminal regions of these SU glycoproteins are exposed and immunogenic in their native state. Examination of equine humoral immune reactivity to the C_C domain of EIAV gp90 disclosed the presence of two equine B-cell epitopes. The carboxyl-terminal residues (peptide 12, amino acids 409 to 434) reacted with 100% of the equine immune sera tested and exhibited the highest level of relative serological reactivity of all of the EIAV gp90 and gp45 peptides evaluated to date (7). The second immunoreactive peptide in the C_C region overlaps peptide 12 by four amino acids and reacted with 85% of the equine immune sera (Table 2). It is interesting that the carboxyl terminus of HIV-1 gp120 also contains a highly conserved and immunodominant antigenic determinant that is strongly reactive with nonneutralizing antibody (45).

Equine serological reactivity to synthetic peptides derived from the V region of EIAV gp90 displayed a lower reactivity than did peptides corresponding to conserved sequences. However, four B-cell determinants have been localized to variable segments of EIAV gp90. It is not clear whether the positive immune reactivities of these variable sequences

reflect antibody binding to the conserved residues in that region. Nevertheless, as with HIV-1 gp120 (16), immunoreactive peptides that have been shown to exhibit sequence variation have been mapped to regions of the EIAV SU glycoprotein (46). Interestingly, a peptide from the V region of EIAV gp90 has been proposed to be functionally homologous to the immunodominant HIV-1 V3 loop (23, 36). Some reported characteristics of the HIV-1 V3 loop include the ability to elicit type-specific neutralizing antibodies in goats, rabbits, chimpanzees, and mice (33, 44), to evoke T-cell proliferation (44), and to contain a major histocompatibility complex class I-restricted cytotoxic T-cell epitope (55). In the case of EIAV, peptide V1 (residues 181 to 210) exhibits 75% serological reactivity with equine immune sera and contains the type-specific neutralization determinant gp90-D_{NT} (2). A portion of peptide V1 (amino acids 198 to 207) is predicted to fold as an amphipathic helix based on the predictive algorithm AMPHI (32), which suggests the presence of a potential helper T-cell epitope (6). Finally, utilizing the Rothbard and Taylor (51) method of predicting cytotoxic T-cell epitopes, a potential cytotoxic T-cell epitope was also identified in this region; located at residues 203 to 206 is the characteristic linear pattern of a charged residue followed by two hydrophobic residues and a polar or charged residue (Lys-Trp-Leu-Glu). Taken together, these properties suggest that the EIAV PND may be an important component of candidate vaccines.

Examination of the serological reactivity of the longitudinal equine sera has allowed the dissection of the early humoral immune response in EIAV experimental infections and how that response evolves over a time span of 5 months. Sera from those ponies inoculated with a virulent EIAV strain generally reacted at a higher absorbance than did sera from animals inoculated with the avirulent prototype strain (Table 4). This difference in antibody responses probably reflects the more vigorous virus replication and higher levels of viremia in animals inoculated with the virulent virus strain. Nonetheless, those ponies infected with the avirulent prototype EIAV still developed antibodies, albeit at lower titers, to the various regions of the SU glycoprotein. Thus, low levels of virus replication appear to be sufficient to stimulate an adequate B-cell response and, as noted previously (53), to induce protective immunity. In agreement with the serological reactivity of the equine immune sera, all six of the longitudinal immune serum panels strongly reacted with peptide 12. Given that antibodies directed against peptide 12 are detected early in experimentally infected horses regardless of the inoculum and remain strongly positive over time, we propose that peptide 12 could be useful as a diagnostic antigen. In contrast, early antibodies were not detected against most of the variable peptides (Table 4). Obviously, positive serological reactivity with peptides corresponding to the V domain reflects a maturation or broadening of the immune response as the chronically infected animal is exposed to multiple, sequential, antigenically distinct viral isolates. In addition, the lack of antibodies to variable sequences during acute and early chronic equine infectious anemia and the presence of antibodies to these variable sequences in asymptomatic carriers suggest that these antibodies to the V domain may contribute to the eventual immunological management of disease and virus replication.

All three of the neutralizing MAbs have been mapped to discrete segments in the V region which overlap with immunogenic peptides that are also recognized by the horse (Fig. 2). The neutralizing MAb epitopes gp90-E_{NT} and gp90-D_{NT} have been localized to adjacent sequences in the PND domain, while the third neutralizing MAb epitope, gp90-C_{NT}, has been mapped 57 amino acids downstream in a hypervariable (V_H) segment of the V domain (46). These data support the model of two distinct neutralizing domains in the V region of EIAV gp90, as deduced previously from competitive ELISA with the panel of MAbs (21), and may prove beneficial in the development of a subunit vaccine. The localization of the neutralizing epitopes to variable sequences supports the concept that antigenic variants can arise by their ability to escape neutralization during a persistent infection. We are currently generating a panel of peptide-specific immune serum in ponies to assay further for neutralizing sites in EIAV gp90 that may not be represented in our panel of neutralizing MAbs.

We have recently developed a detailed structural model of EIAV gp90 (2, 36) based on the predictive algorithms of information theory (14), neural network (13), Bayes statistics (50), Chou and Fasman (8), and AMPHI (32). The structural model presented in Fig. 3 concurs with the parameters of each predictive method and the proposed general retrovirus SU model (12). With the current detailed serological analysis of EIAV gp90, we can now postulate relevant correlations between structure and antigenic properties. Like most surface-exposed proteins, EIAV gp90 is portrayed as an overall globular molecule that is generally hydrophilic with a random distribution of negative and positively charged amino acids. The V domain extends for over 200 residues, encompassing the central, most exposed region of gp90, which is predicted to project out from the surface of the envelope protein. It is noteworthy that the neutralizing MAb epitopes overlap sequences which are predicted to be highly accessible and that the PND is portrayed in the central, most exposed region of the V domain (Fig. 3). The 35-amino-acid V_H region within the V domain is calculated to fold in a beta-sheet–turn–beta-sheet structural motif which could allow the hypervariable sequence to protrude from the envelope protein as an exposed and highly antigenic loop. It has been shown that the most variant sequences in HIV-1 gp120 correlate with well-delineated loops (30), perhaps indicating that a looped structural motif exposes the corresponding sequences to enhanced immune selective pressures. It has also been shown that when all five of the V_H regions of HIV-1 gp120 were experimentally deleted, the resulting antigen was incapable of eliciting neutralizing antibodies (18). The neutralizing MAb epitope gp90-C_{NT} has been mapped to the V_H region of EIAV gp90.

The amino-terminal (peptide 1) and carboxyl-terminal (peptide 12) sequences of EIAV gp90 have been identified as immunodominant determinants displaying 100% reactivity with immune sera from both natural and experimental infections. Both of these peptides are predicted to fold as amphipathic helices, which suggests that the dual-natured alpha helices might play a critical role as helper T-cell epitopes in addition to their role as B-cell determinants. These conserved amphipathic helices may also contribute to oligomer formation by either ionic or hydrophobic interactions along the elongated helix.

Significant structural and functional similarities between the envelope glycoproteins of EIAV and HIV-1 have been previously noted despite the lack of substantial sequence homology (11, 52). The present characterization of immunogenic segments of EIAV gp90 continues to reveal structural and functional homologies between the respective SU glycoproteins of the two lentiviruses. Linear, nonglycosylated antigenic determinants have been localized throughout the

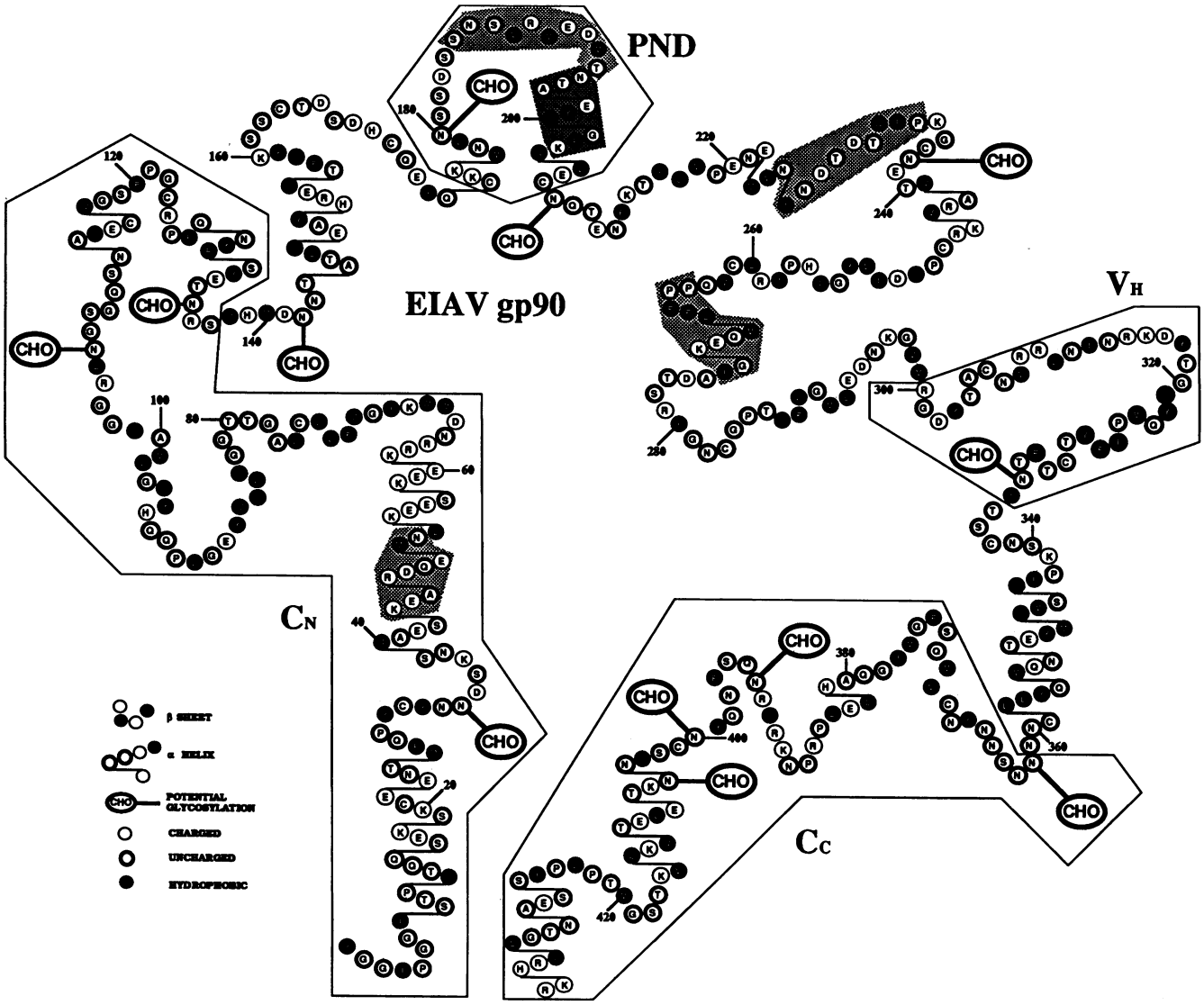

FIG. 3. Structural model of EIAV gp90. A hypothetical structural model of the EIAV SU glycoprotein, gp90, was developed from a battery of predictive computer algorithms (2, 12, 36). The potential structural motifs are indicated in the projected linear amino acid sequence of gp90. Alpha helices are shown as modified helical nets with alternating three and four amino acids per turn connected by single lines. Potential N-linked glycosylation sites are shown as stick figures. Neutral amino acid residues are depicted as incompletely filled circles, charged/polar residues are depicted as open circles, and hydrophobic amino acids are depicted as solid circles. The C_N, C_C, V, and V_H domains are as defined by Payne et al. (46). A region that appears to be functionally similar to the HIV-1 V3 loop has been designated the PND. The localized MAb epitopes are depicted as shaded sequences.

sequence of EIAV gp90, paralleling the identification of B-cell epitopes throughout the sequence of HIV-1 gp120 (16). In both EIAV and HIV-1, neutralizing epitopes have been localized to variable sequences, resulting in a type-specific immune response (19, 33). Both SU glycoproteins contain highly conserved, immunodominant epitopes at the amino- and carboxyl-terminal sequences (44). Finally, pronounced structural propensities in analogous positions on the SU molecules have been identified.

Despite the rapid production of EIAV antigenic variants that are capable of escaping established immune responses, most EIAV-infected animals eventually bring virus replication under immunological control. The data presented here constitute an important component of elucidating the nature of these protective immune responses, but an equally detailed analysis of cellular immune responses to EIAV infection is necessary and under way. It is hoped that the results of these studies will not only produce an effective EIAV vaccine but also provide an important model in designing and evaluating vaccine strategies for infections caused by HIV and other lentiviruses.

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