Analysis of Equine Humoral Immune Responses to the Transmembrane Envelope Glycoprotein (gp45) of Equine Infectious Anemia Virus

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Defined segments of the transmembrane envelope glycoprotein (gp45) of equine infectious anemia virus were expressed as TrpLE fusion proteins and examined for their reactivity in Western immunoblots against a diverse panel of equine immune sera. The most immunogenic region of gp45 was localized to its amino terminus, positioned between the hydrophobic fusion and the transmembrane domains. A series of overlapping synthetic peptides were used in enzyme-linked immunosorbent assays to define an immunodominant epitope within this region. In contrast, the carboxy-terminal half of gp45 displayed both weak and variable immunoreactivity with equine immune sera.

Equine infectious anemia virus (EIAV), a member of the lentivirus subfamily of retroviruses, establishes a persistent infection in horses which results in a chronic disease state typically characterized by periodic episodes of fever and viremia (25, 26, 35). The cyclic nature of the disease is attributed to rapid mutations in the viral envelope gene which give rise to virus variants capable of escaping host immune responses (9, 22, 24, 31). Most infected horses mount a vigorous immune response whose cumulative effect results in the eventual control of the chronic stage of the disease. An integral component of this immune response involves the development of neutralizing antibodies which broaden in their virus specificity throughout the course of infection (30). This clinical course of disease following EIAV infection is unique among the lentiviruses, since host infections with the ungulate lentiviruses (visna virus, maedi virus, and caprine arthritis-encephalitis virus) or immunodeficiency lentiviruses (human [HIV], simian [SIV], and feline immunodeficiency viruses) usually result in a long latent or inapparent disease state which gradually progresses to a degenerative stage that is ultimately fatal (25). Consequently, the dynamic nature of the disease and the unique outcome of EIAV infection provide a suitable model for examining host and viral factors which may be relevant to the immunological control of persistent lentivirus infections in other host systems.

Since the humoral immune response of the host is known to play a significant role in affecting the clinical course of the disease, we have been examining those peptide components of the viral structural proteins that are immunogenic during persistent infection with EIAV. Previously, we characterized the B-cell determinants present in the conserved and variable peptide sequence domains of the EIAV surface envelope glycoprotein (16, 23) as well as the immunogenic domains of the major core protein, p24 (3a). As an extension of these previous studies, we performed a detailed serological analysis of the EIAV transmembrane envelope glycoprotein (gp45) to determine which segments are important as

B-cell determinants during persistent infection of the host with EIAV.

The various overlapping gene segments of gp45 derived from the Wyoming cell-adapted strain of EIAV which were subcloned and expressed as TrpLE fusion proteins in *Escherichia coli* as previously described are shown in Fig. 1 (12, 13, 15, 23). Before their characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting, lysates from induced cultures were enriched for gp45 *env* fusion proteins by urea extraction of insoluble protein aggregates. Each *env* fusion protein was characterized for correct size by SDS-PAGE analysis (11), and the identities of the *env* fusion peptides were determined by their reactivities in Western blots to a rabbit polyclonal antiserum prepared against high-performance liquid chromatography (HPLC)-purified gp45 (not shown) (1a, 2). To detect *env* fusion proteins which were reactive with antibody to gp45 in blots, the membranes were incubated with ¹²⁵I-labeled protein A as previously described (23, 31). The level of expression observed for the various *env* fusion proteins was both size and sequence dependent, with smaller segments (up to 150 amino acids) being expressed at the highest levels and larger segments containing hydrophobic sequences from the transmembrane domain of gp45 expressed at significantly lower levels. These results are consistent with the observations by others that retrovirus transmembrane domains and hydrophobic sequences in general are difficult to express in *E. coli* (36, 37).

To identify regions of the transmembrane envelope glycoprotein that are immunogenic during persistent EIAV infection, recombinant *env* fusion proteins expressed from 17 gp45-coding gene fragments (Fig. 1) were reacted in Western blots against a panel of equine immune sera; the results are summarized in Table 1. Immune sera were obtained from Shetland ponies experimentally infected with a standard inoculum of a virulent isolate derived from the cell-adapted Wyoming strain of EIAV (12, 20; E1 through E5 in Table 1) or from horses naturally infected with uncharacterized field strains of EIAV (N6 through N20 in Table 1). Sera were identified as positive (infected) or negative (uninfected; normal horse sera [NHS]) for EIAV infection on the basis of

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FIG. 1. Expression map of EIAV gp45. DNA restriction fragments from various segments of the EIAV gp45 *env* gene which were cloned and expressed as TrpLE fusion proteins are shown by the heavy dark lines above the map. Cloned restriction fragments which terminate with *Hpa*I or *Eco*RI contain noncoding sequences at their 3' termini (thin solid lines). All sequences expressed from the carboxy terminus of gp45 should terminate translation at the TAG codon shown. One of the expression clones encodes 44 amino acids from the carboxy terminus of gp90 (HdD). The two stippled boxes represent the putative fusion domain (FD) and transmembrane segment (TM), and the open box represents the 3' long terminal repeat (LTR). A segment of predominantly charged amino acids (RYLKKKFHHKHASRED) located in a similar position in HIV-1 gp41 is shown as (+/-). The five potential N-linked glycosylation sites are indicated by the solid vertical bars. Abbreviations for the DNA restriction sites are as follows: A, *Alu*I; Ap, *Ap*aI; D, *Dra*I; Hc, *Hinc*II; Hd, *Hind*III; Hp, *Hpa*I; N, *Nru*I; R, *Eco*RI; S, *Sal*I; Sp, *Spe*I; U, *Sau*3A.

standard immunoassays (34). All of the sera tested reacted with purified gp45 (Table 1), confirming our previous results which identified a conserved and immunodominant domain within the transmembrane envelope glycoprotein among antigenically variant strains of EIAV (23). Nearly all of the immune sera reacted with recombinant fusion proteins containing *env*-encoded sequences from the region between the hydrophobic fusion and transmembrane domains at the amino terminus of gp45. In contrast, *env* fusion proteins containing sequences downstream of the transmembrane domain from the carboxy-terminal half of gp45 displayed both weak and variable reactivity with the same panel of immune sera. Four of the immune sera (N8, N9, N10, and N18 [Table 1]) did not recognize any *env* sequences from this region despite uniform serological reactivity with sequences from the amino terminus of gp45. To further illustrate the organization of gp45 into discrete immunogenic halves, the serological reactivity results from Table 1 were summarized and presented graphically in Fig. 2.

To further identify the *env* sequences which constitute the immunodominant B-cell determinant contained within the 68-amino-acid segment defined by fusion proteins HcHc and SA (Table 1), overlapping synthetic peptides from this region of gp45 (Fig. 3, peptides R31 through R34 and 51) whose sequence and relative positions are outlined in the general conformational model proposed by Gallaher et al. for viral transmembrane proteins (5) were prepared. Two additional

peptides, one from the putative fusion domain at the amino terminus of gp45 (FP) and a second from a potential monoclonal antibody (MAb) binding site (45-B), were also synthesized. Peptide synthesis, purification, and characterization were performed essentially as described for EIAV p26 antigenic analysis (3a). Each peptide was reacted against the panel of equine immune sera by using a modified enzyme-linked immunosorbent assay (ELISA) which was optimized for use with peptide antigens (1). To establish negative control values, each peptide was reacted against a panel of NHS in an ELISA, and the absorbance values were averaged. Values for immune sera which recognized peptides in ELISA were expressed in terms of relative reactivity compared with values obtained for the NHS panel, and the results are summarized in Table 2. A value of 0 indicates no serological reactivity with the peptide. Values of 1 through 4 represent absorbances equal to or greater than two, four, six, or eight times the average absorbance obtained with NHS, respectively. The most frequent serological reactivities were observed with peptides R32 and 51, but only peptide R32 reacted with 100% of the sera tested. The strongest relative reactivity was also observed with peptide R32, with 75% of the positive sera registering a maximum value of 4. The observed immunoreactivity rapidly decreased to 55 to 60% when peptide sequences adjacent to R32 and 51 were examined (Fig. 3).

Previous studies had characterized two nonneutralizing

TABLE 1. Reactivity of recombinant EIAV gp45 antigens with equine immune sera in Western blots

Horse serum	Reactivity with antigen ^a																	
	TrpLE	gp45	Amino terminal								Carboxy terminal							
			D	HpSp	SS	HcHc	SA	HcU	HcN	DS	UU	ApS	ApR	NS	NSp	NR	SD	SpHp
NHS	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
E1	—	+	+	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
E2	—	(+)	+	+	+	+	+	+	+	(+)	(+)	(+)	(+)	(+)	+	+	(+)	—
E3	—	(+)	+	+	+	+	+	+	+	—	(+)	(+)	+	(+)	+	+	+	—
E4	—	(+)	+	+	(+)	+	+	(+)	(+)	—	—	—	—	—	—	—	—	—
E5	—	+	+	+	+	+	+	+	+	(+)	(+)	(+)	+	(+)	+	+	+	—
N6	—	+	+	+	+	+	+	+	+	(+)	—	(+)	+	(+)	+	+	(+)	—
N7	—	+	+	+	(+)	+	+	+	(+)	(+)	—	+	+	—	—	—	—	—
N8	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
N9	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
N10	—	(+)	+	+	(+)	+	+	+	(+)	—	—	—	—	—	—	—	—	—
N11	—	+	+	+	+	+	+	+	+	(+)	—	—	(+)	—	(+)	(+)	—	—
N12	—	+	+	+	+	+	+	+	+	(+)	—	—	(+)	—	(+)	(+)	—	—
N13	—	+	+	+	+	+	+	+	+	(+)	—	(+)	(+)	(+)	(+)	(+)	—	—
N14	—	+	+	+	+	+	+	+	+	+	—	—	+	—	—	+	—	+
N15	—	+	+	+	+	+	+	+	+	+	—	—	(+)	—	(+)	(+)	(+)	(+)
N16	—	+	+	+	+	+	+	+	+	(+)	—	—	(+)	—	(+)	(+)	(+)	—
N17	—	(+)	+	+	+	+	+	+	+	(+)	—	—	+	—	+	+	(+)	—
N18	—	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	—	—	—	—	—	—	—	—	—
N19	—	+	+	+	+	+	+	+	+	—	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
N20	—	+	+	+	+	+	+	+	+	—	(+)	(+)	+	—	(+)	+	(+)	(+)

^a Antigens consisted of HPLC-purified gp45, the TrpLE protein, and recombinant gp45 fusion proteins as defined in Fig. 1 and the legend thereto. Reactivities in Western blot analyses are designated positive [+], weak positive [(+)], and negative [—].

MAbs which define distinct epitopes (45-A and 45-B) present in gp45 (9). One epitope, 45-A, had previously been mapped to a highly conserved and immunodominant segment of about 90 amino acids present within the amino terminus of gp45 (23). To further localize and identify the *env* sequences involved in the recognition and binding of these MAbs, each MAb was reacted in both Western blots and ELISAs against selected *env* fusion proteins and the panel of synthetic peptides described in Table 2. Reactivity with peptide R33 localized the minimum epitope for 45-A to the seven amino acids within the proposed disulfide-bonded loop at the apex of the extended helix shown in Fig. 3. More than half of the

sera from persistently infected horses either failed to react with this peptide or reacted very weakly in ELISAs (Table 2). The epitope for 45-B was localized to a segment of 30 amino acids within the proposed cytoplasmic domain of gp45 on the basis of its reactivity with the fusion protein NS (Fig. 1) in Western blots (not shown). The 45-B epitope is immediately adjacent to but does not include the segment of highly charged amino acids shown as (+/-) in Fig. 2. Although the segment of gp45 which contains the MAb epitope 45-B is recognized as a B-cell determinant in mice, it is weakly immunogenic in horses during persistent infection with EIAV (Table 2).

FIG. 2. Immunogenic domains of EIAV gp45. The reactivity of a panel of 20 equine immune serum specimens against 16 TrpLE-gp45 *env* fusion proteins by Western blot analyses is summarized from the data in Table 1. The ratios of positive serological reactivities, as localized to the respective segments of the transmembrane envelope glycoprotein, to the total number of sera are shown in parentheses above the open boxes. The two hydrophobic segments of gp45, FD and TM (fusion domain and transmembrane segment, respectively), are shown (▨). The segment of highly charged residues (+/-) described in the legend to Fig. 1 is indicated (■). The relative positions of the two MAb binding sites, 45-A and 45-B, are shown (□) above the map. Predicted B-cell epitopes as determined by computer analysis of the gp45 *env* sequence are shown above the map as either major (▼) or minor (▽) determinants according to the parameters set by the computer program (21). aa, Amino acids.

FIG. 3. Proposed conformational model of EIAV gp45. The amino-terminal, extracytoplasmic domain of the transmembrane envelope glycoprotein of EIAV (gp45) is shown; the model is based on calculated structural parameters and the serological reactivity data presented in this study (5). The peptides used in the ELISA which define the immunodominant determinant are outlined and numbered as in Table 2, and the percentage of sera reactive with each peptide is shown in parentheses. Peptide R33 defines a minimum MAb binding site, gp45-A. FP represents a peptide from the proposed fusion domain at the amino terminus of the transmembrane glycoprotein.

A final analysis of the EIAV transmembrane envelope glycoprotein involved a comparison of its antigenicity as determined from its observed serological reactivity with its predicted antigenicity as determined by computer analysis of the gp45 sequence (21). The immunogenic domains, as determined from the results of this study and shown in Fig. 2, show poor correlation with those sequences of gp45 predicted to contain major B-cell determinants. The computer analysis predicted the major B-cell epitopes to be clustered within the proposed intracellular domain of gp45

(Fig. 2) which was shown in this study to be weakly immunogenic in EIAV-infected horses. In contrast, the amino terminus of gp45, which was demonstrated to be extremely immunogenic in EIAV-infected horses, was predicted to be weakly antigenic (Fig. 2). The only accurate correlation involved the lack of predicted B-cell determinants within the carboxy-terminal 75 amino acids of gp45 and the poor immunoreactivity observed with fusion proteins from this region (Fig. 2). These results suggest that the computer programs used in this study for the sequence

TABLE 2. Reactivity of immune horse sera with EIAV gp45 synthetic peptides by ELISA

Horse serum ^a	Reactivity with synthetic peptide ^b :						
	FP (446-465)	R31 (512-524)	R32 (522-534)	51 (534-547)	R33 (537-543)	R34 (545-557)	45-B (672-700)
E1	0	0	4	2	0	0	4
E2	0	0	3	3	0	0	2
E3	0	0	4	3	0	0	1
E4	1	0	2	1	0	0	0
E5	1	1	4	4	1	1	2
N6	0	0	4	0	0	0	1
N7	1	4	4	3	1	1	4
N8	2	4	4	2	1	2	2
N9	1	4	4	1	1	3	1
N10	0	0	4	0	0	0	0
N11	1	0	4	2	0	1	1
N12	1	3	4	2	1	1	2
N13	1	0	4	4	1	3	0
N14	1	4	4	2	2	1	0
N15	3	3	4	4	1	2	1
N16	0	2	4	1	0	0	0
N17	1	2	2	1	0	1	1
N18	1	0	3	1	0	1	1
N19	2	1	4	3	0	0	0
N20	3	3	1	3	1	1	1

^a Equine immune sera were obtained essentially as described in the text.

^b Numbering in parentheses corresponds to amino acid residues of the EIAV *env* gene product, with residue 1 being the initiator methionine of gp90 (29). ELISA values are presented as relative peptide reactivities as described in the text.

analysis of transmembrane envelope proteins may fail to correctly predict which epitopes are normally immunogenic in the host because of conformational considerations or the partitioning of peptide segments into extracellular or intracellular domains.

As has been observed for HIV type 1 and type 2 (HIV-1 and HIV-2) and SIV, an immunodominant epitope within the amino terminus of the transmembrane envelope glycoprotein of EIAV has been localized to a pair of closely spaced and highly conserved cysteine residues (7, 8, 10, 27). The epitopes also contain or are adjacent to glycine residues which frequently are associated with reverse turns or bends in protein secondary structure (4). Polypeptide chain reversals associated with reverse turns are usually found at exposed areas of the protein surface and as such may be representative of antigenic sites (14). A nearly identical structural motif is also present within the amino-terminal halves of many retroviral transmembrane proteins (5), and serological analyses using fusion proteins containing this segment of the human T-cell lymphotropic virus type I envelope suggest the presence of a major immunogenic determinant (32). For both gp41 of HIV-1 and gp45 of EIAV, it was shown that the presence of both cysteine residues was important for full immunological reactivity of a peptide from this region (7). Two adjacent peptides, R32 and 51, define the major antigenic sites in gp45 of EIAV, with R32 representing the immunodominant site (Fig. 3). In this respect, the antigenic organization of EIAV gp45 most closely resembles that reported for HIV-1 gp41, which also contains a second major antigenic site in proximity to the primary immunodominant epitope (18, 19).

Although the biological significance of these conserved structural determinants among diverse transmembrane glycoproteins is unknown, their apparent association with regions of dominant antigenicity may provide us with the means to rapidly identify epitopes useful in the diagnosis of persistent retroviral infections. The identification of immunodominant epitopes within the transmembrane envelope

glycoproteins of the primate lentiviruses has led to the use of recombinant *env* fusion proteins and synthetic peptides in the diagnosis of AIDS (3, 10, 19, 33). Synthetic peptides have also been utilized as sensitive and specific antigens for the detection of antibodies to HIV-1 and HIV-2 and in site-directed serological tests to help distinguish infections by the respective viruses (6, 17, 18). In the case of EIAV, recombinant fusion proteins or synthetic peptides from the immunodominant domain of gp45 may serve as ideal sources of diagnostic antigen because of the minimal amount of sequence variation found between antigenic variants within this region (22). On the basis of this serological study with a limited panel of equine immune sera, it is likely that a synthetic peptide of 26 amino acids (residues 522 to 547) would react strongly with the majority of sera from persistently infected horses. In addition to their role as major B-cell determinants, these conserved regions of the viral transmembrane glycoproteins have also been associated with the antibody-mediated enhancement of infectivity, as observed for HIV and SIV (27a), and suppression of lymphoproliferation by retroviruses (28). Whether this segment of EIAV gp45 is associated with similar pathological processes remains to be established.

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