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Major Species-Specific Antibody Epitopes of the *Ehrlichia chaffeensis* p120 and *E. canis* p140 Orthologs in Surface-Exposed Tandem Repeat Regions▽

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***Ehrlichia chaffeensis* and *E. canis* have a small subset of tandem repeat (TR)-containing protein orthologs, including p120/p140, which elicit strong antibody responses. The TR regions of these protein orthologs are immunoreactive, but the molecular characteristics of the p120/p140 epitopes have not been determined. In this study, the immunodeterminants of the *E. chaffeensis* p120 and *E. canis* p140 were identified and molecularly defined. Major antibody epitope-containing regions of both p120 and p140 were localized to the TR regions, which reacted strongly by Western immunoblotting with antibodies in sera from *E. chaffeensis*-infected dogs or patients and *E. canis*-infected dogs, respectively. Single continuous species-specific major epitopes within the *E. chaffeensis* p120 and *E. canis* p140 TRs were mapped to homologous surface-exposed glutamate/aspartate-rich regions (19 to 22 amino acids). In addition, minor cross-reactive epitopes were localized to homologous N- and C-terminal regions of p120 and p140. Furthermore, although the native and recombinant p120 and p140 proteins exhibited higher-than-predicted molecular masses, posttranslational modifications were not present on abnormally migrating p120 and p140 TR recombinant proteins as determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry.**

Ehrlichia chaffeensis and *Ehrlichia canis* are obligately intracellular bacteria that exhibit tropism for mononuclear phagocytes (22). Recently, a number of studies have demonstrated that antibodies play an essential role in immunity against ehrlichia pathogens (5, 23, 24, 26). Furthermore, a small subset of *E. chaffeensis* and *E. canis* proteins react strongly with antibodies in sera from infected humans or dogs and thus are considered to be major immunoreactive proteins (2, 3, 13, 19). Molecularly characterized major immunoreactive proteins of *E. chaffeensis* and *E. canis* include four protein ortholog pairs (p200/p200, p120/p140, p47/p36, and VLPT/p19) (4, 10, 11, 15–17). Three of these ortholog pairs (p120/p140, p47/p36, and VLPT/p19) have acidic serine-rich tandem repeats (TRs), and continuous species-specific epitopes have been identified in the TRs of p47/p36 and VLPT/p19 (4, 10, 15, 16).

p120 is differentially expressed by dense-cored *E. chaffeensis* and is found on the surface of the organism and free in the morula space; however, the role of this protein in pathobiology or in eliciting a protective immune response is unknown (18). *E. chaffeensis* p120 has two to five nearly identical serine-rich 80-amino-acid TRs, and similarly orthologous *E. canis* p140 contains 12 or 14 nearly identical serine-rich 36-amino-acid TRs (25, 28, 30, 31). Previous studies demonstrated that the TR regions of the p120 and p140 proteins were immunoreactive (16, 27, 30); however, the specific molecular immunodeterminant(s) was not defined.

Determining the molecular characteristics of ehrlichial immunodeterminants involved in eliciting a humoral immune response during infection is important for understanding the molecular basis of immunity to *Ehrlichia* species. In this study, we mapped and molecularly defined a single major continuous species-specific antibody epitope in the repeat unit of *E. chaffeensis* p120 and *E. canis* p140 and identified two homologous minor epitope-containing regions in the N- and C-terminal regions of the proteins that elicit cross-reactive antibodies.

MATERIALS AND METHODS

Culture and purification of ehrlichiae. *E. chaffeensis* (Arkansas strain) and *E. canis* (Jake strain) were propagated and purified by size exclusion chromatography as previously described (12, 20). The fractions containing bacteria were frozen and utilized as antigen and DNA sources.

Preparation of *Ehrlichia* genomic DNA and antigen. Genomic DNA and antigen were purified from *E. chaffeensis* (Arkansas strain) and *E. canis* (Jake strain) as previously described (14). *Ehrlichia*-infected DH82 cell culture supernatants (0.5 ml) were collected 5 days postinfection without disturbing the cell monolayer and clarified by high-speed centrifugation (10,000 × *g* for 5 min) to remove ehrlichiae. Supernatants were subsequently concentrated 10-fold using a Microcon ultracentrifugal filter with a 10-kDa cutoff (Millipore, Billerica, MA).

PCR amplification of the *Ehrlichia* genes. Oligonucleotide primers for the amplification of the *E. chaffeensis* p120 and *E. canis* p140 gene fragments were designed manually or by using PrimerSelect (Lasergene v5.08; DNAStar, Madison, WI) according to the sequences in GenBank (accession numbers U49426 and NC_007354, respectively) and synthesized (Sigma-Genosys, Woodlands, TX) (Table 1). Gene fragments corresponding to the N termini (p120N/p140N), the C termini (p120C/p140C), and the whole open reading frames (p120W/p140W) were amplified by PCR (Fig. 1A). Constructs containing the tandem repeat regions (designated p120TR and p140TR, respectively, in this report) were described previously and used in this study (27, 30). The *E. chaffeensis* p120TR contained only the first two tandem repeats (R1 and R2), whereas the p140TR contained the complete tandem repeat region (14 repeats) of the *E. canis* p140 (Fig. 1A).

PCR was performed with PCR HotMaster mix (Eppendorf, Westbury, NY)

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TABLE 1. Oligonucleotide primers for amplification of the *E. chaffeensis* p120 and *E. canis* p140 gene fragments

Fragment	Primer		Amplicon size (bp)
	Name	Sequence (5′ to 3′)	
p120	p120N-F	ATGGATATTGATAATAGTAACATAAGTAC	1,644
	p120C-R	TACAATATCATTTACTACATTGTGATT	
p120N	p120N-F	ATGGATATTGATAATAGTAACATAAGTAC	162
	p120N-R	TGTGTCATCTTCTTGCTCTTG	
p120C	p120C-F	ATTCTAGTAGAAGATTTGCCATTAG	444
	p120C-R	TACAATATCATTTACTACATTGTGATT	
p140	p140N-F	ATGGATATTGATAACAATAATGTGACTAC	2,064
	p140C-R	TATTAAATCAACTGTTTCTTTGTTAGT	
p140N	p140N-F	ATGGATATTGATAACAATAATGTGACTAC	183
	p140N-R	TGGATTTCCTACATTGTCATTC	
p140C	p140C-F	GAAGTACAGCCTGTTGCAG	324
	p140C-R	TATTAAATCAACTGTTTCTTTGTTAGT	

and the appropriate *Ehrlichia* genomic DNA as the template. The thermal cycling profile was 95°C for 3 min, 30 cycles of 94°C for 30 s, annealing temperature (1°C less than the lowest primer melting temperature) for 30 s, and 72°C for the appropriate extension time (1 min/1,000 bp), followed by a 72°C extension for 10 min and a 4°C hold.

Expression and purification of the recombinant *Ehrlichia* p120 and p140 proteins. The amplified PCR products were cloned directly into the pBAD/Thio-TOPO expression vector (Invitrogen, Carlsbad, CA) and transformed into *Escherichia coli* TOP10 cells (Invitrogen). The resulting transformants were screened by PCR for correctly oriented inserts, and plasmids from the positive transformants were isolated and sequenced to verify the inserts with an ABI Prism 377XL DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Texas Medical Branch Protein Chemistry Core Laboratory. Recombinant protein expression was performed for 4 h after induction with 0.2% arabinose, and proteins were purified under native conditions using HisSelect columns (Sigma, St. Louis, MO). The recombinant TR regions of *Ehrlichia* p120 and p140 were expressed as glutathione *S*-transferase (GST) fusion proteins as previously described (27, 30).

p120 and p140 synthetic peptides. For the *E. chaffeensis* p120, five overlapping peptides corresponding to a single repeat unit (p120R-N, p120R-I1, p120R-I2, p120R-I3, and p120R-C) were commercially synthesized (Bio-Synthesis, Lewisville, TX) (Fig. 1B, left panel; see also Fig. 5A, below, for sequences). Fine mapping within the p120R-I1 region was performed with four overlapping peptides (p120R-I1-S1, p120R-I1-S2, p120R-I1-S3, and p120R-I1-S4; Bio-Synthesis) (Fig. 1B, left panel; see also Fig. 5A for sequences). For p140, six overlapping peptides (p120R-1 to p120R-6) corresponding to the different regions of the *E. canis* p140R were synthesized (Bio-Synthesis) (Fig. 1B, right panel; see also Fig. 6A, below, for sequences). All peptides were supplied as lyophilized powders and resuspended in molecular biology-grade water (1 mg/ml).

Antisera. Sera from two convalescent anti-*E. chaffeensis* dog (nos. 2251 and 2495) sera and one convalescent anti-*E. canis* dog (no. 2995) serum were obtained from experimentally infected dogs. Sera from dogs exhibiting clinical signs or hematologic abnormalities consistent with canine monocytic ehrlichiosis were submitted to the Louisiana Veterinary Medical Diagnostic Laboratory from veterinarians statewide and screened by immunofluorescence assay (IFA), as described previously (12). Human monocytotropic ehrlichiosis (HME) patient sera were kind gifts from Focus Technologies (Cypress, CA) and William Nicholson at the Centers for Disease Control and Prevention (Atlanta, GA). Rabbit anti-p120 and anti-p140 antisera were generated against synthetic keyhole limpet hemocyanin-conjugated peptides located in the epitope-containing region of each respective repeat unit (p120, SKVEQEETNPEVLIKDLQDVAS; p140, EHSSSEVGEKVSKTSKEESTPEVKA) by a commercial vendor (Bio-Synthesis).

Gel electrophoresis and Western immunoblotting. Purified *E. chaffeensis* or *E. canis* whole-cell lysates or recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, and Western immunoblotting was performed as previously described (13), except that primary dog sera were diluted 1:100, human sera were diluted 1:200, and rabbit antisera were diluted 1:1,000.

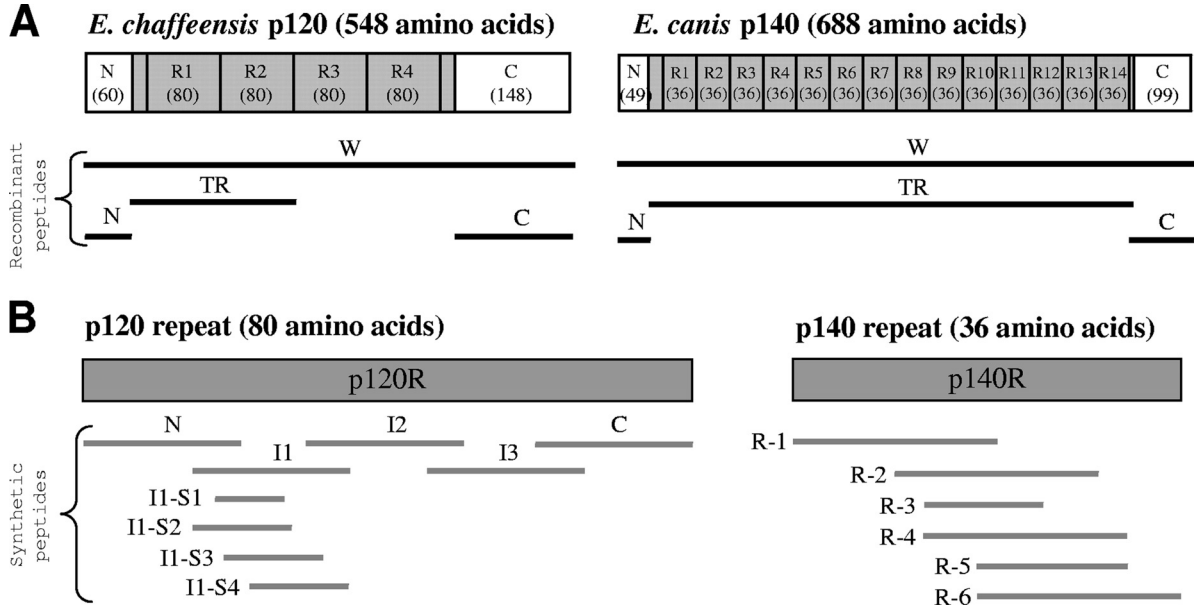

FIG. 1. (A) Schematic of *E. chaffeensis* p120 and *E. canis* p140 proteins showing domains, location of TRs (number of amino acids in parentheses; R = repeat), and recombinant proteins used for epitope mapping. For both p120 and p140, there were two incomplete repeats preceding the first repeat and following the last repeat, respectively, which were homologous to tandem repeats and these are also shown in gray. The N terminus, C terminus, TR region, and whole protein (W) are shown. (B) Schematic of synthetic peptides used to map the tandem repeat epitope of *E. chaffeensis* p120 and *E. canis* p140 proteins.

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TABLE 2. Predicted and observed molecular masses and amino acid analysis results for *E. chaffeensis* p120 and *E. canis* p140 proteins

Protein	Molecular mass (kDa)[a]			No. (%) of residues that are:		
	Predicted	Observed	MALDI-TOF[c]	Glutamate	Serine	Valine
E. chaffeensis p120						
p120	77.1	110	ND[d]	96 (17.5)	67 (12.2)	59 (10.8)
p120 N	22.3	23	ND	2 (4.0)	4 (8.0)	1 (2.0)
p120TR[b]	47.0	58	47.1	78 (22.3)	52 (14.8)	40 (11.4)
p120 C	33.0	33	ND	16 (10.8)	11 (7.4)	18 (12.2)
Native p120	60.8	95/75	ND			
E. canis p140						
p140	89.9	140	ND	120 (17.4)	109 (15.8)	89 (12.9)
p140 N	21.5	22	ND	4 (6.6)	6 (9.8)	7 (11.5)
p140 TR	85.6	130	85.9	111 (21.4)	96 (18.5)	69 (13.3)
p140 C	28.3	28	ND	5 (4.6)	7 (6.5)	13 (12.0)
Native p140	73.6	125	ND			

[a] Including the fusion tags; all were thioredoxin (16.3 kDa) except for p120TR and p140TR (GST tag; 28 kDa).
[b] Only the first two repeats were cloned and expressed, but the amino acid content values are for the whole repeat region.
[c] As determined by MALDI-TOF mass spectrometry of the recombinant protein.
[d] ND, not determined.

ELISA. Enzyme-linked immunosorbent assay (ELISA) plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated (0.5 μg/well; 50 μl) with recombinant proteins or synthetic peptides suspended in phosphate-buffered saline (pH 7.4). Proteins and peptides were absorbed for 1 h at room temperature with gentle agitation, and subsequently washed thrice with 200 μl Tris-buffered saline containing 0.2% Tween 20 (TBST). Plates were blocked with 100 μl 10% equine serum (Sigma) in TBST for 1 h at room temperature with agitation and washed. Convalescent dog or human sera diluted (1:100 or 1:200, respectively) in 10% equine serum–TBST were added to each well (50 μl) and incubated at room temperature for 1 h with gentle agitation. The plates were washed four times, and 50 μl alkaline phosphatase-labeled goat anti-dog or human immunoglobulin G (H+L) secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted (1:5,000) in 10% equine serum–TBST was added and incubated for 1 h at room temperature. The plates were washed four times, and substrate (100 μl; BluePhos; Kirkegaard & Perry Laboratories) was added to each well. The plates were incubated in the dark for 30 min with agitation, color development (A_{650}) was determined on a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA), and data were analyzed by using SoftmaxPro v4.0 (Molecular Devices). Optical density (OD) readings represent the mean OD for three wells (± standard deviations) after subtracting the OD value of the buffer-only wells. A reading of >0.2 OD units above the negative control absorbance was considered positive for all samples. In addition, a reading of 0.2 to 0.5 OD units above the control absorbance was considered a weak positive, and a reading of >0.5 OD units above the control absorbance was considered a strong positive.

Mass spectrometry. Sample solution or a protein standard (1 μl) was spotted directly onto a matrix-assisted laser desorption ionization (MALDI) target plate and allowed to air dry. Sinapic acid (Aldrich, Milwaukee, WI) matrix solution (1 μl; 50:50 acetonitrile/water) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Decon Laboratories, King of Prussia, PA) before inserting into the mass spectrometer. Mass spectrometry was performed using a MALDI–time-of-flight (MALDI-TOF) mass spectrometer (4800 MALDI TOF/TOF proteomics analyzer; Applied Biosystems) at the University of Texas Medical Branch Mass Spectrometry Core Laboratory. Data were acquired with the software package including 4000 series Explorer (v3.6 RC1; Applied Biosystems). The instrument was operated in positive ion linear mode, with a mass range as required. A total of 4,000 laser shots were acquired and averaged from each sample shot. External calibration was performed using cytochrome *c* or bovine serum albumin according to the target molecular weight.

Sequence analysis. Amino acid sequence alignments of *E. chaffeensis* p120 and *E. canis* p140 were performed with MegAlign (Lasergene v5.08; DNAStar). The major epitopes of p120 and p140 were examined for sequence similarities with other proteins by using the protein-protein basic local alignment search tool (BLAST [http://www.ncbi.nlm.nih.gov/BLAST]).

Statistics. Statistical differences between experimental groups were assessed with the two-tailed Student's *t* test, and significance was indicated by a *P* value of <0.05.

RESULTS

***E. chaffeensis* p120 and *E. canis* p140 composition and characteristics.** In the *E. chaffeensis* (Arkansas strain) p120 and *E. canis* (Jake strain) p140 proteins, glutamate (17.5% in p120 and 17.4% in p140), serine (12.2% and 15.8%, respectively), and valine (10.8% and 12.9%, respectively) were the most frequently occurring amino acids (Table 2). Moreover, in the TRs of p120 and p140, the occurrences of these three residues (E, S, and V) were more frequent (22.3%/21.4%, 14.8%/18.5%, and 11.4%/13.3%, respectively). On the contrary, in the N and C termini of p120 and p140, the occurrences of these three residues became less frequent, except for the valine content in the C terminus of p120. Due to the large proportion of glutamate residues, the p120 and p140 proteins were highly acidic (pI 3.8 and 3.9, respectively).

Amino acid sequence similarity within the N terminus and surface-exposed motif of the repeat region between *E. chaffeensis* p120 and *E. canis* p140 has been reported (16, 30), but sequence similarity within the C terminus and analyses of specific regions have not been fully explored. The amino acid identity was ~50% for the first 32 amino acids of the N terminus. Similarly, homologous (~39% amino acid identity) regions were identified in the C terminus of p120 and p140 (Fig. 2). A BLAST search determined no substantial sequence similarity with other known ehrlichial proteins or proteins from organisms in closely related genera.

Identification of the native *E. chaffeensis* p120 and *E. canis* p140 proteins. Western blotting identified two strongly reactive native proteins with molecular masses of ~95 kDa and ~75 kDa (both larger than the predicted mass of 61 kDa, which was based on the amino acid sequence) and a few less prominent proteins (75 to 50 kDa) in *E. chaffeensis* whole-cell lysates and culture supernatants that reacted with monospecific rabbit antiserum against the synthetic p120R-I1 peptide; however, this antiserum did not react with any proteins in *E. canis* whole-cell lysates (Fig. 3A). Similarly, a native protein with a molecular mass of ~125 kDa (larger than the predicted mass of 74 kDa) and a few smaller and less prominent proteins

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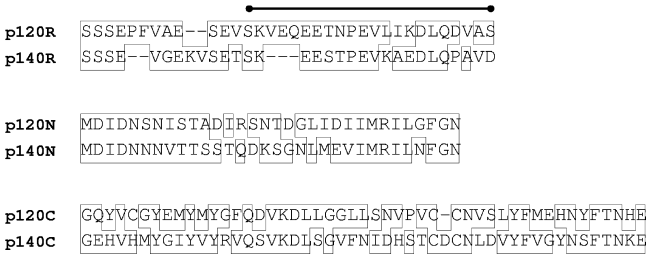

FIG. 2. Alignments of amino acid sequences of homologous regions in the tandem repeat unit and N- and C-terminal regions of *E. chaffeensis* p120 and *E. canis* p140 proteins. Residues that match the consensus within two distance units are boxed, and gaps are shown by dashes. The major TR epitopes of *E. chaffeensis* p120 (22-mer) and *E. canis* p140 (19-mer) are identified with a bar.

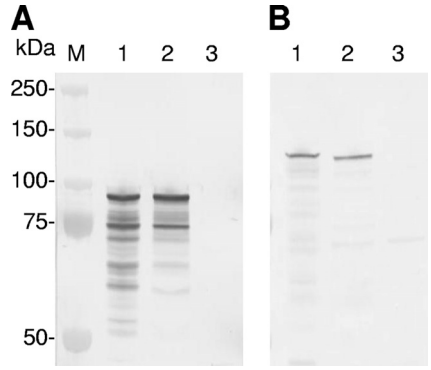

FIG. 3. Identification of native *E. chaffeensis* p120 and *E. canis* p140 proteins by Western immunoblotting. (A) *E. chaffeensis* whole-cell lysates (lane 1), supernatants derived from *E. chaffeensis*-infected cells (lane 2), and *E. canis* whole-cell lysates (lane 3) reacted with rabbit anti-p120R-I1 antibody. (B) *E. canis* whole-cell lysates (lane 1), supernatants derived from *E. canis*-infected cells (lane 2), and *E. chaffeensis* whole-cell lysates (lane 3) reacted with rabbit anti-p140 peptide antibody. Preimmunization rabbit serum controls did not recognize *Ehrlichia* whole-cell lysates (data not shown). M, Precision protein standard (Bio-Rad).

in *E. canis* whole-cell lysates reacted with monospecific rabbit antiserum against a p140R peptide. Proteins in *E. chaffeensis* whole-cell lysates did not react with this antiserum (Fig. 3B). Preimmunization rabbit serum controls did not react with proteins in *E. chaffeensis* or *E. canis* whole-cell lysates by Western immunoblotting (data not shown).

Epitope mapping of *E. chaffeensis* p120 and *E. canis* p140 with recombinant proteins. To conclusively determine the major epitope-containing regions of p120 and p140, the recombinant full-length p120 and p140 proteins (p120W/p140W) and fragments corresponding to three distinct domains, including the N terminus (p120N/p140N), tandem repeat region (p120TR/p140TR), and C terminus (p120C/p140C), were expressed (Fig. 1A). The p120W/p140W and p120TR/p140TR recombinant proteins exhibited molecular masses substantially larger than predicted by their amino acid sequences by SDS-PAGE. In contrast, the recombinant p120N/p140N and p120C/p140C exhibited masses consistent with those predicted by their amino acid sequences. MALDI-TOF mass spectrometry determined that the molecular masses of recombinant p120TR and p140TR proteins were nearly identical to those predicted by the corresponding amino acid sequences (Table 2), and thus the abnormal migration was not associated with posttranslational modifications.

By Western immunoblotting, the recombinant p120W and p120TR reacted very strongly with two anti-*E. chaffeensis* dog sera derived from two dogs (nos. 2251 and 2495) experimentally infected with *E. chaffeensis* and sera from two HME patients (nos. SC07 and CDC4) that had detectable *E. chaffeensis* antibodies by IFA; however, recombinant fragments of the p120N and p120C did not react or reacted weakly with the dog or patient sera (Fig. 4A). Similarly, recombinant p140W and p140TR reacted very strongly with three anti-*E. canis* dog sera derived from an experimentally infected dog (no. 2995) and two naturally infected dogs (nos. 2160 and 4283); however, recombinant p140N and p140C did not react or reacted weakly with those dog sera (Fig. 4B). These human or dog sera did not recognize thioredoxin or GST proteins, and the normal human or dog sera did not recognize these recombinant proteins by Western immunoblotting (data not shown).

Peptide mapping of the major immunodeterminants of *E. chaffeensis* p120 and *E. canis* p140. To localize the major epitope(s) of *E. chaffeensis* p120 protein, five overlapping peptides (p120R-N, p120R-I1, p120R-I2, p120R-I3, and p120R-C) spanning the repeat unit of p120 (Fig. 1B [left panel] and 5A) were detected by ELISA with the anti-*E. chaffeensis* dog serum (no. 2251) and three HME patient sera (nos. 3, 18, and 20) that demonstrated *E. chaffeensis* antibodies by IFA. Four peptides (p120R-N, p120R-I2, p120R-I3, and p120R-C) were not immunoreactive or weakly immunoreactive with only one serum, but p120R-I1 (22-mer) located in the N-terminal region of the p120R reacted strongly with all sera by ELISA (Fig. 5B to E). Furthermore, peptides p120R-N and p120R-I2, which contain amino acids (SKVEQEETNP and DLQDVAS, respectively) present in the N and C termini of the p120R-I1 (22-mer) and p120-S1 (EQEETNPEVLIK), representing a central overlapping region, were not reactive with antibodies individually; however, collectively the peptide p120-I1 (SKVEQEETNPEV LIKDLQDVAS) reacted strongly with antibodies in sera, suggesting that 22 amino acids were necessary for full constitution of the p120 TR epitope (Fig. 5A to E). Additional mapping with smaller peptides (p120R-I1-S1, S2, S3, and S4) demonstrated a significant contribution (S1, S3, and S4 [$P < 0.05$ for all sera]; S2 [$P < 0.05$ for all patient sera]) by both N-terminal (SKV) or C-terminal (DLQD) amino acids of peptide p120R-I1 and indicated that the continuous epitope was represented by this peptide (Fig. 5A to E).

To identify the peptide sequence containing the immunodeterminant in *E. canis* p140 protein, six overlapping peptides (designated p140R-1 to p140R-6 from the N terminus to C terminus) spanning the repeat unit of p140 (Fig. 1B, right panel, and 6A) were reacted with four anti-*E. canis* sera from naturally infected dogs (nos. 2160, 6, 10, and 18) (Fig. 6B to E). By ELISA, all overlapping peptides except for peptide p140R-3 (11-mer) reacted with anti-*E. canis* dog sera. Peptide p140R-4 (19 amino acids; SKEESTPEVKAEDLQPAVD), which was predicted to be surface exposed and overlapped with the identified *E. chaffeensis* p120 epitope (see above and Fig. 2), had significantly ($P < 0.05$) stronger immunoreactivity with the majority of sera tested by ELISA. Additional peptide mapping with overlapping peptides (p140-R1) demonstrated that

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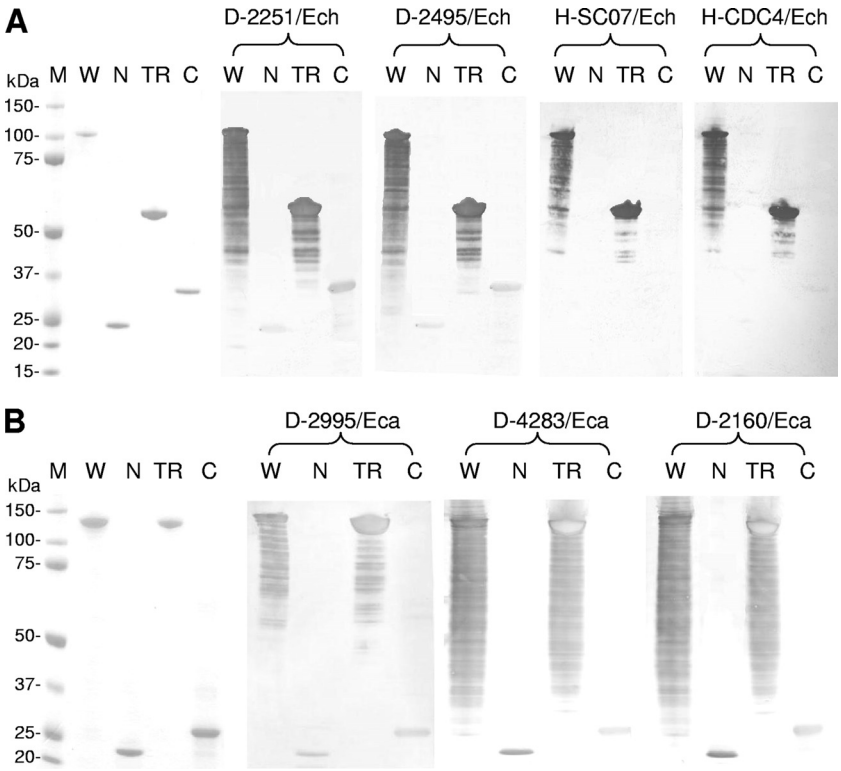

FIG. 4. Immunoreactivity of recombinant proteins of *E. chaffeensis* p120 and *E. canis* p140 by Western immunoblotting. (A) SDS-PAGE and total protein staining of purified recombinant p120 recombinant fragments (whole protein [W], N terminus, TRs [two repeats], and C terminus) (left) and the corresponding Western immunoblot probed with two anti-*E. chaffeensis* dog sera (experimentally infected animals, nos. 2251 and 2495 [D-2251/Ech and D-2495/Ech]) and two HME patient sera (nos. SC07 and CDC4 [H-SC07/Ech and H-CDC4/Ech]) (right). (B) SDS-PAGE and total protein staining of purified recombinant p140 protein fragments (whole protein [W], N terminus, TR [14 repeats], and C terminus) (left) and corresponding Western immunoblot probed with three anti-*E. canis* sera from one experimentally infected dog (no. 2995 [D-2995/Eca]) and two naturally infected dogs (nos. 4283 and 2160 [D-4283/Eca and D-2160/Eca]) (right). Human or dog sera did not recognize thioredoxin or GST proteins, and the normal human or dog sera did not recognize these recombinant proteins by Western immunoblotting (data not shown). M, Precision protein standard (Bio-Rad).

the N-terminal amino acids (SKEESTP) of p140-R4 did react with antibodies and contributed to the epitope, as p140-R4 exhibited consistently stronger immunoreactivity than p140R-5, which lacked amino acids SKEES (Fig. 6A to E). Furthermore, peptide p140R-4, which contained additional C-terminal amino acids (EDLQPAVD) compared to p140R-3, exhibited strong immunoreactivity, whereas p140R-3 lacking these amino acids was virtually nonreactive, indicating a dominant contribution associated with these residues (EDLQPAVD) to the epitope. Comparative immunoreactivity between peptides p140R-2 and R-4 indicated that additional C-terminal amino acid residues, AVD, also contributed significantly ($P < 0.05$) to epitope reactivity with half of the dog sera examined (Fig. 6A to E).

Identification of immunoreactive regions for cross-reaction between *E. chaffeensis* p120 and *E. canis* p140. To examine cross-reactions between p120 and p140 and to localize the regions containing the cross-reactive epitope(s), the recombinant p120 and p140 proteins corresponding to three distinct domains (N terminus, TR region, and C terminus) were reacted with the anti-*E. canis* dog sera and anti-*E. chaffeensis* dog or patient sera. By Western immunoblotting, the recombinant p120TR and p140TR proteins did not react or reacted weakly with heterologous anti-*E. canis* sera and anti-*E. chaffeensis* sera, respectively; however, either recombinant N or C termini of the p120 and p140 proteins did cross-react with heterologous sera (Fig. 7).

DISCUSSION

It is well established that tandem repeat-containing proteins of *Ehrlichia* spp. are primary targets of the humoral immune response and elicit vigorous and, in many instances, species-specific antibodies (4, 10, 16). *E. chaffeensis* p120 and *E. canis* p140 protein orthologs are well-characterized major immunoreactive proteins strongly recognized by sera from HME patients and *E. canis*-infected dogs (16, 28, 30). Although previous studies demonstrated that *E. chaffeensis* p120 and *E. canis* p140 proteins reacted with antibodies in dog and/or patient sera (12, 27, 29, 30), the immunologic properties of these two proteins were not fully defined, and the extent of the host response directed against them has remained undetermined.

All of the major immunoreactive TR proteins of *E. chaffeensis* and *E. canis* that have been characterized, including p120 and p140 orthologs, are highly acidic due to a predominance of glutamate/aspartate; moreover, they also appear to be serine rich, which usually occurs more frequently within TRs of these proteins (4, 10, 11, 15, 16). Interestingly, major continuous

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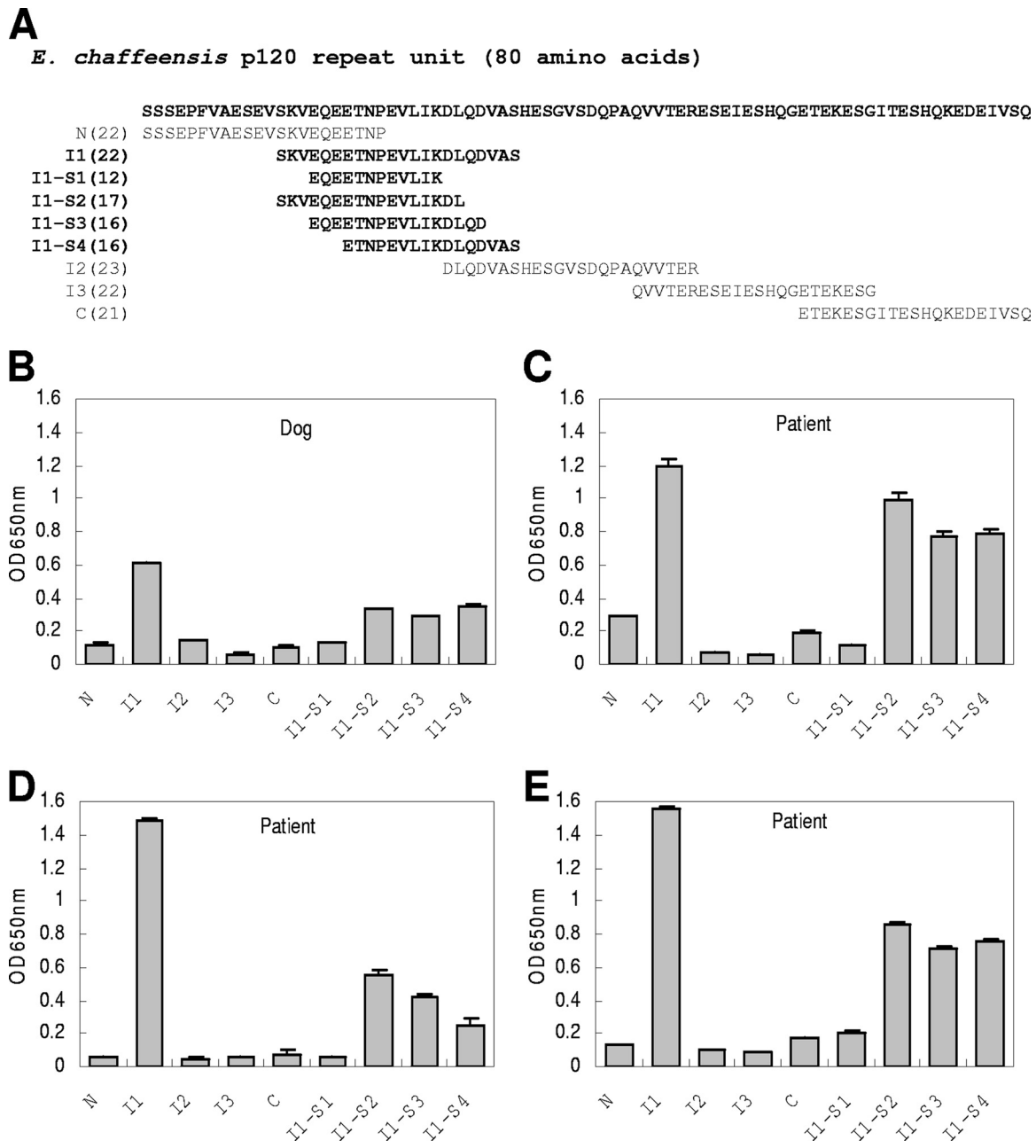

FIG. 5. Immunoreactivity of overlapping synthetic peptides spanning the *E. chaffeensis* p120 repeat unit by ELISA. (A) Sequence and orientation of all overlapping peptides representing the *E. chaffeensis* p120 repeat unit. (B) *E. chaffeensis* p120 peptides reacted with the anti-*E. chaffeensis* dog serum derived from an experimentally infected dog (no. 2251). (C to E) *E. chaffeensis* p120 peptides reacted with sera from three HME patients (nos. 3, 18 and 20, respectively). The OD readings represent the means for three wells (±standard deviations) with the OD of the buffer-only wells subtracted. The OD readings of peptide p120R-I1 were significantly higher than those of smaller overlapping peptides (I1-S1, I1-S3, and I1-S4, $P < 0.05$ for all sera; I1-S2, $P < 0.05$ for all patient sera). Normal dog or human serum did not recognize these peptides (data not shown).

antibody epitopes of these proteins have been mapped to serine-rich acidic domains (4, 10, 15–17), which indicates a relationship between these domains and the host immune response; however, the specific role of these amino acids in directing the immune response against *Ehrlichia* is still unknown. The major epitope-containing regions of both *E. chaffeensis* p120 and *E. canis* p140 protein orthologs were mapped to the serine-rich tandem repeat units, which is consistent with the location of epitopes in other ehrlichial TR-containing proteins. The antibody epitopes in p120TR and p140 TR, which exhibited the strongest antibody reactivity with both dog and human sera, were localized to the p120R-I1 (22 amino acids) and p140R-4 (19 amino acids) regions, respectively, which are homologous and predicted to be surface-exposed domains. Therefore, consistent with the location of epitopes mapped in other TR ehrlichial proteins, the con-

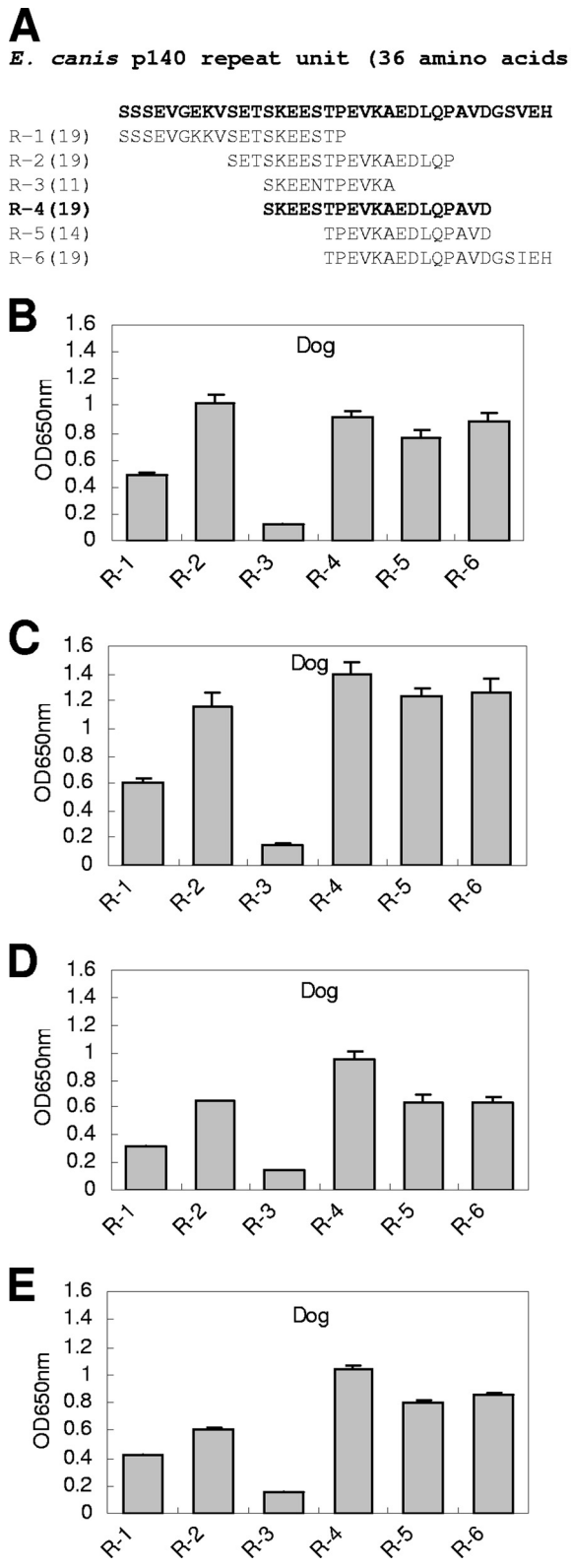

FIG. 6. Immunoreactivity of *E. canis* p140 repeat overlapping synthetic peptides as determined by ELISA. (A) Six overlapping peptides spanning the *E. canis* p140 repeat unit. (B to E) *E. canis* p140 peptides reacted with anti-*E. canis* dog sera obtained from four naturally infected dogs (nos. 2160, 6, 10, and 18, respectively). The OD readings represent the means for three wells (±standard deviations) with the OD of the buffer-only wells subtracted. The OD readings of peptide R-4 were significantly higher than those of R-2 with half of the dog sera (nos. 10 and 18, $P < 0.05$). The normal dog serum did not recognize these peptides (data not shown).

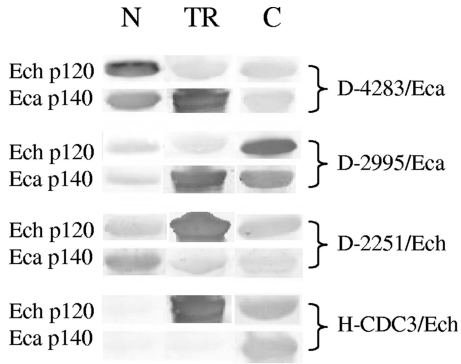

FIG. 7. Localization of minor cross-reactive epitopes between *E. chaffeensis* p120 and *E. canis* p140 proteins by Western immunoblotting. *E. chaffeensis* p120 and *E. canis* p140 recombinant proteins (N terminus, TR, and C terminus) reacted with anti-*E. canis* sera (nos. 4283 and 2995 [D-4283/Eca and D-2995/Eca]) and anti-*E. chaffeensis* sera (nos. 2251 and CDC3 [D-2251/Ech and H-CDC3/Ech]).

served surface-exposed domains of p120 and p140 TRs contained a dominant continuous immunodeterminant.

The lengths of the *E. chaffeensis* p120 and *E. canis* p140 epitopes were similar (~20 amino acids) and consistent in size with that described of other molecularly characterized continuous ehrlichial epitopes, including those of VLPT/p19, p47/36, and p200 (*E. canis*) (4, 10, 15, 17). Although smaller peptides associated with the mapped epitope reacted with antibodies, significantly higher antibody reactivities were observed with peptides consisting of ~20 amino acids, a finding that is consistent with the epitope length we have mapped on other TR proteins and similar in size to a neutralizing continuous antibody epitope consisting of 15 amino acids recently mapped in the *Helicobacter* UreB protein (8). However, a smaller 6-amino-acid continuous epitope has been mapped in *Anaplasma marginale* msp1a (1). Although major continuous epitopes have been mapped on several ehrlichial TR proteins, one conformational epitope has been mapped in VLPT (10), and there may be other discontinuous epitopes associated with these major immunoreactive proteins that were not determined in this study. However, the host response to the continuous epitopes is strong and consistent with the response observed with recombinant folded proteins, suggesting the absence of dominant conformational epitopes.

Unlike other immunoreactive protein orthologs of *Ehrlichia*, the major epitopes of p120 and p140 do exhibit some sequence similarity, raising the possibility of eliciting cross-reactive antibodies; however, antibodies generated against epitope-containing peptides did not cross-react by Western immunoblotting, indicating that these epitopes appear to be primarily species specific, a finding consistent with a previous study using antisera against recombinant p120TR and p140TR (16). Hence, the cross-reactive immune response elicited by *Ehrlichia* spe-

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cies does not appear to be directed against the major continuous antibody epitopes identified thus far in *E. chaffeensis* and *E. canis* TR proteins, including p120/p140. However, we did identify that minor cross-reactive epitopes in the N- and C-terminal regions, which is consistent with the fact that substantial sequence similarity occurs in these regions. Therefore, as we have proposed with major continuous epitopes identified in other ehrlichial TR proteins, the p120/p140 TR epitopes could be utilized for species-specific diagnostic development.

We previously reported that some recombinant ehrlichial immunoreactive proteins exhibited larger-than-predicted masses, similar to their native counterparts, by gel electrophoresis (4, 10, 15, 16), which was also observed in this study with both recombinant and native p120 and p140 proteins. The recombinant p120W/p140W and p120TR/p140TR exhibited abnormally large molecular masses, but the recombinant N- and C-terminal regions (p120N/p140N, p120C/p140C) migrated as expected, indicating that the highly acidic serine-rich TR was responsible for the anomalous electrophoretic behavior of these proteins. This abnormal electrophoretic migration was previously associated with detection of carbohydrate, based on chemical reactivity, suggesting glycosylation of TRs (16). In this study, we determined by mass spectrometry that the molecular masses of p120TR and p140TR were consistent with those predicted by their amino acid sequences; therefore, the glycosylation is not responsible for the larger-than-predicted masses of the p120 and p140 proteins. It is likely that the high acidity of these proteins, particularly in the TR regions, is responsible for the abnormal electrophoretic behavior. This is supported by studies demonstrating that highly acidic proteins exhibit abnormal migration patterns during gel electrophoresis (6, 7). Like p120 and p140 proteins, we recently reported that another major immunoreactive protein (VLPT) of *E. chaffeensis* also exhibited a larger-than-predicted mass on gel, but mass spectrometry determined that this protein was not posttranslationally modified (10). The molecular masses of the native *E. chaffeensis* p120 (~95 kDa) and *E. canis* p140 (~125 kDa) proteins were smaller than previously reported masses (~120 kDa and ~140 kDa, respectively) (16, 30). This difference is likely related to differences in SDS-PAGE procedures and accuracy of molecular mass markers. Nevertheless, the native proteins identified from the ehrlichial lysate by the antibodies against synthetic epitope peptides and the masses of the recombinant p120 or p140 protein (without fusion tag) were in agreement in this study.

The major immunoreactive proteins of *Ehrlichia* spp. have been identified and consist of a small subset of proteins. Three of these proteins in *E. chaffeensis* and *E. canis* are acidic, serine rich, and contain TRs (4, 10, 15, 30). The host immune response appears to be primarily directed at continuous species-specific epitopes within the TRs, which suggests similar characteristics contribute to immune response stimulation and production of species-specific antibodies directed at these TR epitopes. However, the role of continuous major antibody epitopes within ehrlichial TR proteins in eliciting a protective immune response is currently undefined. Although protective antibody epitopes have been mapped to an *E. chaffeensis* major outer membrane protein, p28 (9), new studies indicate that ehrlichial TR proteins are secreted and interact with important host cell targets and facilitate pathogen survival (21). Thus, studies to examine whether the host antibody response elicited by continuous epitopes in TR proteins such as p120/p140 are protective will provide much needed insight into the protective ehrlichial antigens and effective immune responses.

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