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Ehrlichia canis gp200 Contains Dominant Species-Specific Antibody Epitopes in Terminal Acidic Domains▽

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Species-specific antibody epitopes within several major immunoreactive protein orthologs of *Ehrlichia* species have recently been identified and molecularly characterized. In this study, dominant B-cell epitopes within the acidic (pI 5.35) ankyrin repeat-containing 200-kDa major immunoreactive protein (gp200) of *Ehrlichia canis* were defined. The *E. canis* gp200 gene (4,263 bp; 1,421 amino acids) was cloned and expressed as four (N-terminal, 1,107 bp; N-internal, 910 bp; C-internal, 1,000 bp; and C-terminal, 1,280 bp) overlapping recombinant proteins. The N-terminal, C-internal, and C-terminal polypeptides (369, 332, and 426 amino acids, respectively) were strongly recognized by antibody, and the major epitope(s) in these polypeptides was mapped to four polypeptide regions (40 to 70 amino acids). Smaller overlapping recombinant polypeptides (14 to 15 amino acids) spanning these regions identified five strongly immunoreactive species-specific epitopes that exhibited conformational dependence. The majority of the epitopes (four) were located in two strongly acidic (pI 4 to 4.9) domains in the distal N- and C-terminal regions of the protein flanking the centralized ankyrin domain-containing region. The amino acid content of the epitope-containing domains included a high proportion of strongly acidic amino acids (glutamate and aspartate), and these domains appear to have important biophysical properties that influence the antibody response to gp200.

Patients and dogs infected with *Ehrlichia chaffeensis* and *Ehrlichia canis* develop antibodies to a relatively well defined group of proteins that constitute the major immunoreactive proteins of *Ehrlichia* (4, 7, 17, 28). Many of these immunoreactive proteins and their corresponding orthologs have been identified and molecularly characterized in *E. chaffeensis* and *E. canis* (5, 15, 19, 21, 24, 25, 35, 36). The majority of the molecularly characterized immunoreactive proteins are secreted, serine/threonine rich, and strongly acidic and exhibit electrophoretic masses that are substantially larger than those predicted by amino acid sequences (5, 15, 19, 34, 35). Furthermore, the major immunodeterminants have been mapped to acidic serine-rich tandem repeats in many of these proteins (5, 19, 33, 35).

Recently, the largest major immunoreactive ehrlichial protein ortholog (gp200) of *E. canis* and *E. chaffeensis* has been identified and molecularly characterized (15). The recombinant *E. canis* gp200 N-terminal domain (P43) reacts strongly with antibodies in serum from dogs naturally and experimentally infected with *E. canis* (16, 17). The native and recombinant *E. chaffeensis* and *E. canis* gp200 orthologs exhibit molecular masses larger than those predicted by their amino acid sequences but lack serine-rich tandem repeats present in other ehrlichial proteins (15). However, the gp200s have ankyrin domains containing numerous ankyrin repeats (at least 21) that may mediate protein-protein interactions. The function of gp200 is unknown, but the protein is translocated to the nucleus of infected monocytes (23). gp200 exhibits homology with *Anaplasma phagocytophilum* AnkA (3), which is a type IV secretion substrate and is phosphorylated by host Abl-1 and Src tyrosine kinases (8, 13). AnkA also is translocated to the nucleus of infected neutrophils, where it binds DNA and may be involved in modulation of host cell gene transcription (26).

Elimination of *Ehrlichia* infection requires both cellular and humoral immune mechanisms. Although cell-mediated immune mechanisms are critically important in protection from intracellular pathogens, a number of studies have demonstrated an important role for humoral immunity in host defenses against ehrlichial pathogens (7, 30–32). Immunocompetent mice lacking B cells cannot clear a sublethal infection with *Ixodes ovatus*-carried ehrlichiae (32), and adoptive transfer of polyclonal immune serum protects severe combined immunodeficiency (SCID) mice from *E. chaffeensis* challenge (32). Specifically, protection has been demonstrated with antibodies directed against p28 of *E. chaffeensis* (11, 12, 25, 30), and studies with *E. canis* demonstrated that opsonization with antibodies resulted in the intracellular killing of the organism in vitro (10). SCID mice are protected from lethal infection by passive transfer of anti-*Ehrlichia muris* polyclonal antibody, but Fab antibody fragments are not protective (7).

The objective of this study was to define the epitopes involved in antibody recognition of gp200, a well-characterized immunoreactive ehrlichial protein. In this study, we determined that gp200 contains at least five major immunoreactive epitopes, the majority of which were localized to terminal domains dominated by strongly acidic amino acids. These domains appear to have important biophysical properties that influence the antibody response to gp200.

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TABLE 1. Oligonucleotide primers used to PCR amplify regions of *E. canis* gp200 for epitope mapping

Target[a]	Forward primer		Reverse primer		Amplicon size (bp)
	Name	Sequence	Name	Sequence	
gp200					
$Nt_{1–369}$	1f	ATGTCAGATCCAAAACAAGGT	1107r	TCCATCTACAAGTCCAAAATCTAA	1,107
$Ni_{360–663}$	1081f	AATTTAGATTTTGGACTTGTA	1992r	CCCACGCTCACTTGGTAGGT	911
$Ci_{651–983}$	1951f	AATTTTGTTGGGGATTCGTTA	2952r	ACCTGCGTTATTCTTTTGAGTAAG	1,001
$Ct_{980–1406}$	2935f	CAAAAGAATAACGCAGGTGATACA	4221r	ACTAGGAGATGCTGCTTGTTGTTG	1,286
N-terminal region					
$Nt_{8–186}$ (1)	22f	GATCCAGAACAAAATCAAACTA	564r	ACCTAAAACTGCATTCCTAACATCTG	542
$Nt_{8–66}$ (2)	22f	GATCCAGAACAAAATCAAACTA	183r	ATATAAATCTTCACTCTCAGGA	161
$Nt_{66–100}$ (3)	196f	ATGCCTAAGGGTAAAAGAACTGC	300r	TCTTGGCGGTAATGTAGGAGGTAAATC	104
$Nt_{29–72}$ (4)	85f	ATGCAGGAACAAGATCAGCAGCAG	216r	AGCAGCAGTTCTTTTACCCTTAGG	131
$Nt_{8–35}$ (5)	22f	GATCCAGAACAAAATCAAACTA	105r	CTGCTGCTGATCTTGTTCCT	83
$Nt_{86–186}$ (6)	265f	GATGATGAAGATTTACCTCCTACA	564r	ACCTAAAACTGCATTCCTAACATCTG	299
C-internal region					
$Ci_{650–765}$ (7)	1948f	TTAAATTTTGTTGGGGATTCGTT	2295r	ATTCTCACAATTAACATCAACTCCAG	347
$Ci_{757–879}$ (8)	2269f	ACTGGAGTTGATGTTAATTGTGAGAA	2637r	AGATATTGCAGCATAAACCATTGG	368
$Ci_{876–1024}$ (9)	2626f	GCTGCAATATCTGGTAATGAGCA	3072r	GTACCCTTCTTTGTCTCTGGCTGTT	446
$Ci_{757–817}$ (10)	2269f	ACTGGAGTTGATGTTAATTGTGAGAA	2451r	TCCCTTTTCTTTTCTACCTGGAACTATC	182
$Ci_{808–878}$ (11)	2422f	GCGATAGTTCCAGGTAGAAAAGAAAAG	2634r	TATTGCAGCATAAACCATTGGACG	212
$Ci_{862–937}$ (12)	2584f	GTTGAAGTTAATCGAAATAGTGAAATA CGTC	2811r	AGATTTACCAACATCACATCCTTCAGAAA	227
$Ci_{933–985}$ (13)	2797f	GATGTTGGTAAATCTGGAAAAGATGGTA	2955r	ATCACCTGCGTTATTCTTTTGAGTAAGA	158
$Ci_{979–1024}$ (14)	2935f	CAAAAGAATAACGCAGGTGATACACCTT	3072r	GTACCCTTCTTTGTCTCTGGCTGTT	137
$Ci_{876–917}$ (15)	2626f	GCTGCAATATCTGGTAATGAGCA	2751r	TGCTACTGCAACCATAATTAAAGGATTTC	125
$Ci_{915–947}$ (16)	2743f	GCAGTAGCAGATGGTAATGCAGGTCTTC	2841r	AGCATAATGTAACGCTGTATTACCATCTT	98
$Ci_{942–985}$ (17)	2824f	AATACAGCGTTACATTATGCTGTTAGTC	2955r	ATCACCTGCGTTATTCTTTTGAGTAAGA	131
C-terminal region					
$Ct_{1222–1395}$ (18)	3665f	AATACGCGAAATAACTCTGAC	4188r	TACCTGGGTAACTTCTGGTAAAC	523
$Ct_{1222–1274}$ (19)	3665f	AATACGCGAAATAACTCTGAC	3822r	ATCATCACGAATACACTCTG	157
$Ct_{1292–1340}$ (20)	3874f	ATGAAGAAACTTGAGGCACGAG	4020r	GAACGACACAGCACCACTACTTCTTG	146
$Ct_{1222–1340}$ (21)	3665f	AATACGCGAAATAACTCTGAC	4020r	GAACGACACAGCACCACTACTTCTTG	355
$Ct_{1266–1298}$ (22)	3796f	ATGTCTTTATCAGAGTGTATTCGTG	3894r	AGCTCGTGCCTCAAGTTTCTT	98
$Ct_{1332–1371}$ (23)	3994f	ATGGCAAGAAGTAGTGGTGCTGTGTCGTTC	4113r	ATCATTTGACCCAAGACTAGTATCAGA	119
$Ct_{1363–1404}$ (24)	4087f	ATGTCTGATACTAGTCTTGGGTCAAATG	4212r	TCGTGCTTGTTGTTGACTTACAGC	125

[a] Subscripts represent the amino acids contained in the fragment. Numbers in parentheses correspond to those in Fig. 2.

MATERIALS AND METHODS

Preparation of *E. canis* genomic DNA. Genomic DNA was purified from *E. canis* (Jake strain) as previously described (18).

Anti-*E. canis* serum. Convalescent anti-*E. canis* serum was collected from a dog (no. 2995) experimentally infected with *E. canis*.

PCR amplification of *E. canis* gp200 fragments. Oligonucleotide primers were designed to amplify overlapping regions (28 fragments) containing potential *E. canis* gp200 epitopes (Table 1). Amplicons were generated from genomic *E. canis* DNA (HotMasterMix; Eppendorf, Westbury, NY) using the following thermal cycling profile: 94°C for 5 min; 30 cycles of 94°C for 30 s, annealing temperature (5°C less than the lowest primer melting temperature) for 30 s, and 72°C for the appropriate extension time (30 s/500 product base pairs); and 72°C for 7 min.

Recombinant gp200 protein expression and purification. The four largest gp200 amplicons (910 to 1,280 bp), spanning ~99% of the *E. canis* gp200 open reading frame, were cloned into the pUni/V5-His-TOPO Echo donor vector (Invitrogen, Carlsbad, CA). The donor vector is designed to recombine the insert into an acceptor vector with appropriate transcription regulatory and fusion protein coding sequences. The cloned donor vector was transformed into PIR1 *Escherichia coli* (Invitrogen) and selected on LB agar containing kanamycin (50 μg/ml). The resulting transformants were screened by PCR for correctly oriented inserts, and plasmids from the positive transformants were isolated and sequenced to verify proper orientation and frame. Correct donor vectors were recombined by Cre recombinase with the pRSET-E Echo acceptor vector (Invitrogen), which contains a *loxP* recombination site. Recombined vectors were transformed into TOP10 *E. coli* (Invitrogen) for plasmid propagation, and transformants were selected by growth on LB agar with kanamycin (50 μg/ml). Correct orientation and frame of the transformant inserts were verified as described above. These clones and the control plasmid pRSET-E/Uni-CAT (Invitrogen) were transformed into *E. coli* BL21(DE3)pLysS (Invitrogen), and protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to a liquid culture in log growth phase incubated for 3.5 h at 37°C. Following expression, cultures were pelleted at 3,000 × *g* for 20 min and frozen at −80°C.

All smaller recombinant *E. canis* gp200 fragments used for epitope mapping were amplified by PCR, cloned into the pBAD/TOPO ThioFusion expression vector (Invitrogen), and characterized similarly. Expression of the recombinant proteins in TOP10 *E. coli* (Invitrogen) was induced by adding 0.02% arabinose to cultures in log-phase growth. The recombinant *E. canis* gp200 proteins expressed from the recombined pUni/pRSET-E Echo vector were purified under denaturing conditions as described previously (5, 16). The recombinant peptides expressed from the pBAD/TOPO ThioFusion vectors were purified under native conditions as previously described (6). All recombinant proteins were quantitated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Cloning of peptide epitope oligonucleotides. Complementary 42- or 45-bp oligonucleotides encoding all epitopes (14- or 15-mer peptides) (Table 2) were annealed and cloned into the pBAD 102/Directional TOPO ThioFusion expression vector (Invitrogen) as previously described (5).

Gel electrophoresis and protein blotting. Larger recombinant proteins and thioredoxin-fused recombinant proteins and peptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4 to 12% NuPAGE gels (Invitrogen) with MOPS (morpholinepropanesulfonic acid) buffer as previously described (17). Smaller recombinant peptides were separated on 12% NuPAGE gels with MOPS running buffer. For some assays, total protein was visualized in the gel with Imperial Protein Stain (Pierce, Rockford, IL), according to the manufacturer's protocol. Recombinant proteins/peptides were transferred to a nitrocellulose membrane as described previously (17).

Western immunoblotting. Immunoreactivities of recombinant gp200 fragments with anti-*E. canis* dog serum (no. 2995) were determined by Western immunoblotting as previously described (17) with primary antibodies diluted 1:500. Total membrane-bound protein was visualized by Ponceau S staining prior to membrane blocking. Recombinant peptides were detected with anti-V5, anti-

TABLE 2. Complementary oligonucleotides encoding *E. canis* gp200 14- or 15-mer peptides

Region	Oligonucleotide			
	Coding		Noncoding	
	Name	Sequence	Name	Sequence
N-terminal region	pep1-cod	CACCCAGGAACAAGATCAGCAGCAGGGAGCAGTTGGTGGTGCTGTT	pep1-non	AACAGCACCACCAACTGCTCCCTGCTGCTGATCTTGTTCCTG
	pep2-cod	CACCGGTGGTGCTGTTGGTAATAGTCCTATTGAAAGAGAGAGAGTA	pep2-non	TACTCTCTCTCTTTCAATAGGACTATTACCAACAGCACCACC
	pep3-cod	CACCAGAGAGAGAGTAGCTGCTCCTGAGAGTGAAGATTTATATACT	pep3-non	AGTATATAAATCTTCACTCTCAGGAGCAGCTACTCTCTCTCT
	pep4-cod	CACCGATTTATATACTGTGATTATACCTAAGGGTAAAAGAACTGCT	pep4-non	AGCAGTTCTTTTACCCTTAGGTATAATCACAGTATATAAATC
	pep5-cod	CACCAAAAGAACTGCTGCTCCAATTTTGGAAAGAAAGTCTCCTACTCCTGAA	pep5-non	TTCAGGAGTAGGAGACTTTCTTTCCAAAATTGGAGCAGCAGTTCTTTT
C-internal region	pep6-cod	CACCAATACAGCGTTACATTATGCTGTTAGTCATTCAGATAAAGAG	pep6-non	CTCTTTATCTGAATGACTAACAGCATAATGTAACGCTGTATT
	pep7-cod	CACCTCAGATAAAGAGTTTGGTAATAAAGCTATAAAGATATTAATT	pep7-non	AATTAATATCTTTATAGCTTTATTACCAAACTCTTTATCTGA
	pep8-cod	CACCAAGATATTAATTTCACGTAATAGTGTTGGGACTAATAGAGAT	pep8-non	ATCTCTATTAGTCCCAACACTATTACGTGAAATTAATATCTT
	pep9-cod	CACCACTAATAGAGATATTCTTACTCAAAAGAATAACGCAGGTGAT	pep9-non	ATCACCTGCGTTATTCTTTTGAGTAAGAATATCTCTATTAGT
C-terminal region	pep10-cod	CACCCTAGTTAATACGCGAAATAACTCTGACGATACTGTTGCACAT	pep10-non	ATGTGCAACAGTATCGTCAGAGTTATTTCGCGTATTAACTAG
	pep11-cod	CACCACTGTTGCACATTGTGCTCTTTTATCGGATATGAAATATGCT	pep11-non	AGCATATTTCATATCCGATAAAAGAGCACAATGTGCAACAGT
	pep12-cod	CACCATGAAATATGCTCAAAAGATACTTAAATCATGTAACCATGAT	pep12-non	ATCATGGTTACATGATTTAAGTATCTTTTGAGCATATTTCAT
	pep13-cod	CACCTGTAACCATGATACATTAGTGAGAGGAAATAGTAATAATCAA	pep13-non	TTGATTATTACTATTTCCTCTCACTAATGTATCATGGTTACA
	pep14-cod	CACCAGTAATAATCAATCTTTATCAGAGTGTATTCGTGATGATAGTAAA	pep14-non	TTTACTATCATCACGAATACACTCTGATAAAGATTGATTATTACT
	pep15-cod	CACCGATGATAGTAAATATAAAAAAGGTGGAATTTTTAGTAAGTCTTTA	pep15-non	TAAAGACTTACTAAAAATTCCACCTTTTTTATATTTACTATCATC
	pep16-cod	CACCAGAAGTAGTGGTGCTGTGTCGTTCAAACATGTGCAAGAAACAGGA	pep16-non	TCCTGTTTCTTGCACATGTTTGAACGACACAGCACCACTACTTCT
	pep17-cod	CACCCAAGAAACAGGAGTTGACACGTCTGGTCCTTCTGATATAGAAAGT	pep17-non	ACTTTCTATATCAGAAGGACCAGACGTGTCAACTCCTGTTTCTTG
	pep18-cod	CACCGATATAGAAAGTTTAGAGAGATTATCTGATACTAGTCTTGGGTCA	pep18-non	TGACCCAAGACTAGTATCAGATAATCTCTCTAAACTTTCTATATC
	pep19-cod	CACCAGTCTTGGGTCAAATGATTTTGATCAGCGAATGGCAGATTTAGAT	pep19-non	ATCTAAATCTGCCATTCGCTGATCAAAATCATTTGACCCAAGACT
	pep20-cod	CACCGCAGATTTAGATCAAGAAATAGCAAATATTGTTAGTGGTTTACCA	pep20-non	TGGTAAACCACTAACAATATTTGCTATTTCTTGATCTAAATCTGC
	pep21-cod	CACCACTCCTTTACCAGAAGTTACCCAGGTAGCTGTAAGTCAACAACAA	pep21-non	TTGTTGTTGACTTACAGCTACCTGGGTAACTTCTGGTAAACCACT

Xpress, or anti-His (C-terminal) antibodies (Invitrogen) according to the manufacturer's protocol.

Synthetic peptides. Custom peptides (Table 3) were synthesized and purified (Biosynthesis, Inc., Lewisville, TX) and used in an enzyme-linked immunosorbent assay (ELISA) to determine epitope reactivity with anti-*E. canis* serum.

ELISA. The immunoreactivities of recombinant and synthetic peptides were determined as previously described by ELISA (5). A synthetic 20-mer peptide (RNTTVGVFGLKQNWDGSAIS) previously determined to contain the hypervariable region 1 epitope of *E. chaffeensis* p28-19 (kindly provided by Xue-jie Yu, University of Texas Medical Branch) was used as a positive control for immunoreactivity (37). Primary antibody (canine anti-*E. canis* serum) was diluted 1:1,000, and secondary antibody (alkaline phosphatase-labeled goat anti-dog immunoglobulin G [heavy plus light chains]; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 1:2,500. Substrate (Sure Blue; Kirkegaard & Perry) was added, and absorbance was measured with a tunable microplate reader (Molecular Devices, Sunnyvale, CA) at 650 nm. The absorbance of the sample in each well was expressed as the average optical density of three or four wells at 650 nm after subtraction of the thioredoxin-only blank wells.

Sequence analysis of mapped epitopes. *E. canis* gp200 epitopes were examined for homology to other *Ehrlichia* sp. proteins (including gp200 orthologs) using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

gp200 amino acid composition and acidic domains. The overall gp200 (1,421 amino acids) composition was dominated by four hydrophobic (A, I, L, and V), four polar (S, T, N, and Q), two strongly acidic (D and E; 187 amino acids), and two strongly basic (K and R; 144 amino acids) amino acids. Interestingly, a large number ($n = 118$) of glycine (G) residues (high content in fibrous proteins) were also present. Three specific domains were identified according to amino acid composition

TABLE 3. Peptide sequences (14- and 15-mer) spanning the epitope-containing regions of *E. canis* gp200

Epitope region (amino acid positions)	Peptide name	Sequence
N-terminal (29–84)	1	QEQDQQQGAVGGAV
	2	GGAVGNSPIERERV
	3	RERVAAPESEDLYT
	4	DLYTVIIPKGKRTA
	5	KRTAAPILERKSPTP
C-internal (942–985)	6	NTALHYAVSHSDKE
	7	SDKEFGNKAIKILI
	8	KILISRNSVGTNRD
	9	TNRDILTQKNNAGD
C-terminal (1222–1287)	10	LVNTRNNSDDTVAH
	11	TVAHCALLSDMKYA
	12	MKYAQKILKSCNHD
	13	CNHDTLVRGNSNNQ
	14	SNNQSLSECIRDDSK
	15	DDSKYKKGGIFSKSL
C-terminal (1333–1402)	16	RSSGAVSFKHVQETG
	17	QETGVDTSGPSDIES
	18	DIESLERLSDTSLGS
	19	SLGSNDFDQRMADLD
	20	ADLDQEIANIVSGLP
	21	SGLPEVTQVAVSQQQ

and isoelectric point (Fig. 1). The distal terminal (Nt [amino acids 1 to 369] and Ct [last 205 amino acids]) polypeptides exhibited a substantially larger proportion (2:1) of strongly acidic amino acids (D and E) than did the internal region (848 amino acids; positions 370 to 1263) of the protein where ankyrin repeats were located, and the ratio of strongly basic and strongly acidic amino acids was equivalent. Consequently the isoelectric point of the internal ankyrin domain regions was slightly basic (pI 7.4). Although the acidic regions of the protein represented only ~40% of the protein, the highly acidic domains greatly influenced the overall isoelectric point of gp200, resulting in a protein of acidic nature (pI 5.35).

Immunoreactivity of the major *E. canis* gp200 fragments. *E. canis* gp200 was cloned and expressed as four (~1,000-bp) overlapping polypeptides: N-terminal (Nt; amino acids 1 to 369; pI 4.1), N-internal (Ni; amino acids 360 to 663; pI 6.0), C-internal (Ci; amino acids 651 to 983; pI 7.9), and C-terminal (Ct; amino acids 980 to 1406; pI 6.0). The Nt and Ct recombinant gp200 fragments (containing the acidic domains) exhibited substantially larger (~6 kDa)-than-predicted molecular masses by SDS-PAGE (Fig. 2A). Three gp200 recombinant fragments (Nt, Ci, and Ct) reacted strongly with anti-*E. canis* antibody, but the Ni polypeptide exhibited a substantially weaker immunoreactivity (Fig. 2B). The recombinant proteins (Nt, Ni, Ci, and Ct) did not react with healthy dog sera (data not shown). Thus, the three strongly immunoreactive fragments (Nt, Ci, and Ct) were considered to have major B-cell epitopes and were investigated further.

Major epitope-containing region in the Nt polypeptide. The major epitope(s) in the Nt region was identified by evaluating the immunoreactivities of six overlapping recombinant proteins (Fig. 3A). A smaller recombinant protein representing approximately one-half (amino acids 8 to 186) of the Nt region of gp200 was cloned and expressed, and Western blotting revealed that this smaller Nt polypeptide (designated $Nt_{8–186}$) was strongly reactive with dog anti-*E. canis* serum (Fig. 3B). $Nt_{8–186}$ was further divided into five overlapping polypeptides designated $Nt_{8–66}$, $Nt_{66–100}$, $Nt_{29–72}$, $Nt_{8–35}$, and $Nt_{86–186}$. Two polypeptides, $Nt_{8–35}$ and $Nt_{86–186}$, were only weakly immunoreactive with anti-*E. canis* dog serum by Western blotting, while all other fragments were strongly immunoreactive (Fig. 3B). The region from amino acids 36 to 85 was identified in the strongly immunoreactive recombinant polypeptides but not in weakly reactive fragments; thus, this 51-amino-acid section of *E. canis* gp200 was identified as the major epitope-containing region and was located in a highly acidic domain (Fig. 1 and 3C). This polypeptide exhibited high serine/threonine/glutamate/aspartate (STED) content (~31%) (Table 3).

Major epitope-containing region in the Ci polypeptide. The major epitope-containing region within the *E. canis* gp200 Ci polypeptide was identified by evaluating the immunoreactivity of 11 overlapping recombinant polypeptides spanning the Ci major fragment: $Ci_{650–765}$, $Ci_{757–879}$, $Ci_{876–1024}$, $Ci_{757–817}$, $Ci_{808–878}$, $Ci_{862–937}$, $Ci_{933–985}$, $Ci_{979–1024}$, $Ci_{876–917}$, $Ci_{915–947}$, and $Ci_{942–985}$ (Fig. 3A). The five strongly immunoreactive polypeptides in this region, $Ci_{757–879}$, $Ci_{876–1024}$, $Ci_{862–937}$, $Ci_{933–985}$, and $Ci_{942–985}$, spanned amino acids 756 to 1024. However, the major epitope-containing region was identified by excluding the amino acids contained within the remaining polypeptides which were only weakly immunoreactive or not immunoreactive (Fig. 3B). Therefore, the major epitope-containing region was located within amino acids 948 to 978 (Fig. 3C), which also exhibited a high STED content (~29%) (Table 3).

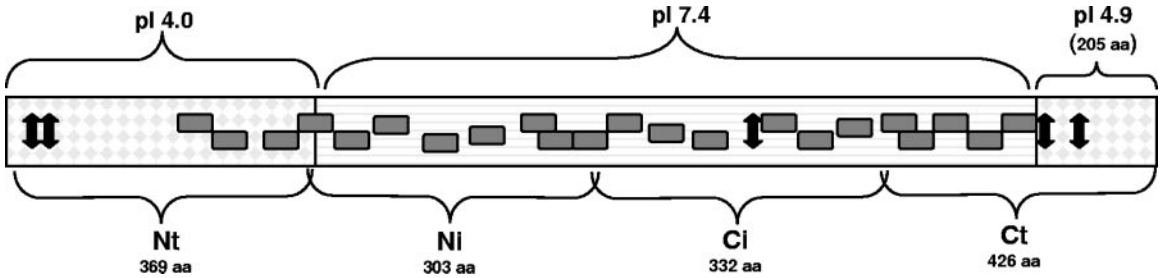

FIG. 1. Schematic of *E. canis* gp200 showing predicted isoelectric points (pIs) of acidic terminal domains and the slightly basic central ankyrin repeat (22 shaded boxes)-containing region. The cloned recombinant expressed regions (Nt, Ni, Ci, and Ct) are shown, and the approximate locations of mapped epitopes are designated by arrows. aa, amino acids.

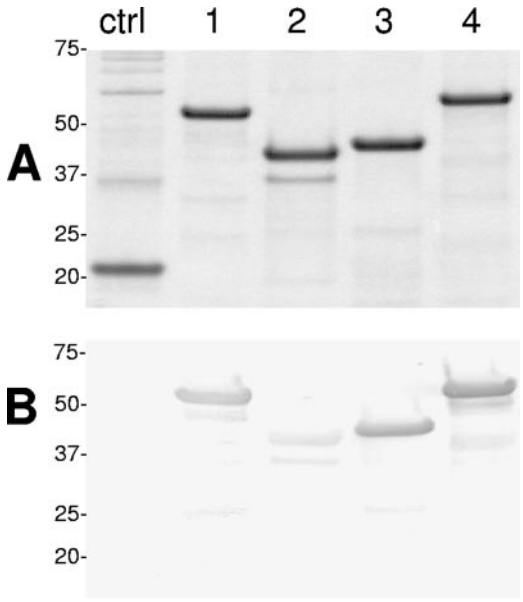

FIG. 2. (A) SDS-PAGE and total protein stain showing four *E. coli*-expressed overlapping *E. canis* gp200 recombinant proteins representing 99% of the open reading frame. Lane 1, Nt; lane 2, Ni; lane 3, Ci; lane 4, Ct. Molecular masses (kilodaltons) are shown on the left. (B) Corresponding Western immunoblot probed with polyclonal dog anti-*E. canis* serum (2995). Lanes and numbers at left are as defined for panel A. Negative controls (ctrl) are recombinant *E. chaffeensis* Dsb (22 kDa) (A) and recombinant chloramphenicol acetyltransferase (28 kDa) (B).

Major epitope-containing region in the Ct polypeptide. The epitope-containing regions in the Ct region of *E. canis* gp200 were identified by evaluating the immunoreactivity of smaller overlapping recombinant proteins (Fig. 3A). The large Ct region was divided roughly in half and expressed as two smaller polypeptides (amino acids 908 to 1203 and 1222 to 1395, respectively). The immunoreactivity of the distal half of the protein was strong, as determined by Western blotting with anti-*E. canis* dog serum (Fig. 3B); conversely, the other half was not immunoreactive (data not shown). The immunoreactive region (designated $Ct_{1222–1395}$) was divided into six overlapping polypeptide fragments: $Ct_{1222–1274}$, $Ct_{1292–1340}$, $Ct_{1222–1340}$, $Ct_{1266–1298}$, $Ct_{1332–1371}$, and $Ct_{1363–1404}$. Four polypeptides, $Ct_{1222–1274}$, $Ct_{1222–1340}$, $Ct_{1266–1298}$, and $Ct_{1363–1404}$, were strongly immunoreactive with anti-*E. canis* antibodies, while two fragments ($Ct_{1292–1340}$ and $Ct_{1332–1371}$) were weakly immunoreactive (Fig. 3B). The epitope-containing regions within the Ct region of *E. canis* gp200 were determined by excluding the amino acids contained by the two weakly immunoreactive recombinant proteins, 1266 to 1340. Therefore, the regions including amino acids 1222 to 1290 and 1372 to 1404 were considered Ct epitope-containing regions and were located in highly acidic domains (Fig. 1 and 3C). Both epitope-containing regions had high STED content, 27% and 24%, respectively (Table 3).

Immunoreactivity of synthetic and recombinant polypeptides. A total of 21 synthetic (14- and 15-mer) and corresponding recombinant overlapping polypeptides were generated to cover the sequences of the four major epitope-containing re-

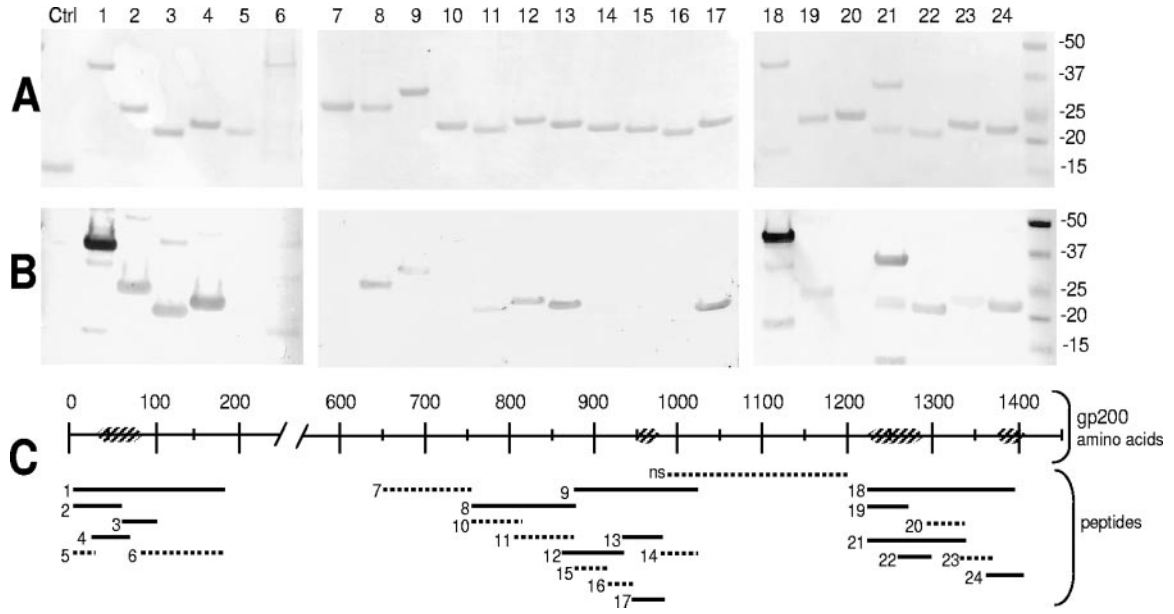

FIG. 3. Immunoreactivities of 24 overlapping recombinant polypeptides covering the major immunoreactive regions of the *E. canis* gp200. Lanes and lines: 1, $Nt_{8–186}$; 2, $Nt_{8–66}$; 3, $Nt_{66–100}$; 4, $Nt_{29–72}$; 5, $Nt_{8–35}$; 6, $Nt_{86–186}$; 7, $Ci_{650–765}$; 8, $Ci_{757–879}$; 9, $Ci_{876–1024}$; 10, $Ci_{757–817}$; 11, $Ci_{808–878}$; 12, $Ci_{862–937}$; 13, $Ci_{933–985}$; 14, $Ci_{979–1024}$; 15, $Ci_{876–917}$; 16, $Ci_{915–947}$; 17, $Ci_{942–985}$; 18, $Ct_{1222–1395}$; 19, $Ct_{1222–1274}$; 20, $Ct_{1292–1340}$; 21, $Ct_{1222–1340}$; 22, $Ct_{1266–1298}$; 23, $Ct_{1332–1371}$; 24, $Ct_{1363–1404}$. Nt, Ci, and Ct represent their locations in the large N-terminal, C-internal, and C-terminal regions of gp200, respectively (subscripts designate the amino acid numbers included in each fragment). (A) Membrane total protein staining. (B) Western immunoblots probed with polyclonal dog anti-*E. canis* serum. (C) Schematic of *E. canis* gp200 illustrating the locations of the 24 recombinant fragments and the epitope-containing regions. Solid lines, strongly immunoreactive recombinant gp200 fragments; dotted lines, weakly immunoreactive or nonimmunoreactive recombinant gp200 fragments; hatched boxes, epitope-containing regions; Ctrl, negative-control recombinant thioredoxin; ns, not shown. Molecular masses (kilodaltons) are shown on the right.

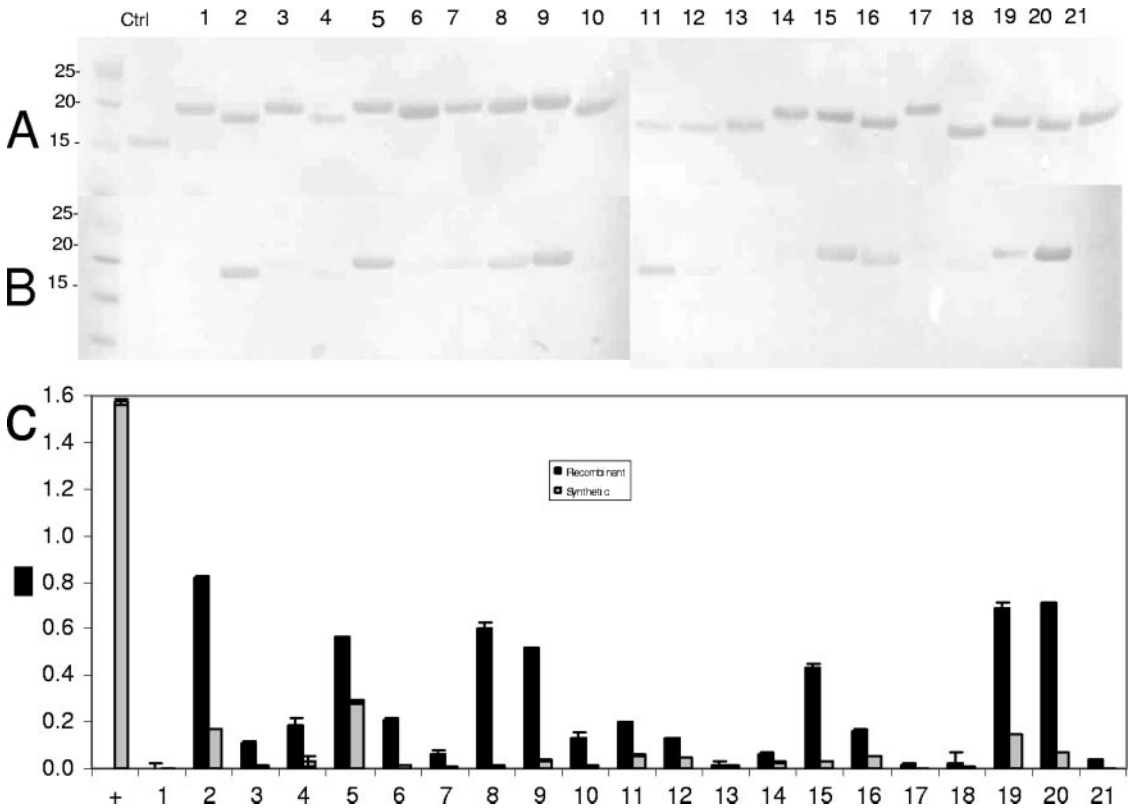

FIG. 4. Immunoreactivities of recombinant and synthetic overlapping 14- and 15-mer peptides (Table 3) spanning the epitope-containing regions of *E. canis* gp200. Positive control (Ctrl or +), *E. chaffeensis* p28-19 peptide epitope; lane and *x*-axis numbers correspond to the peptide numbers in Table 3. (A) Membrane total protein staining. (B) Western immunoblot probed with polyclonal dog anti-*E. canis* serum. (C) Immunoreactivities of the recombinant gp200 peptides and the corresponding synthetic gp200 peptides expressed as the average optical densities at 650 nm of quadruplet samples as determined by ELISA. The recombinant *E. canis* gp200 regions corresponding to the overlapping peptides are labeled under the *x* axis. Molecular masses (kilodaltons) are shown on the left.

gions of *E. canis* gp200. Recombinant thioredoxin-fused peptides with amino acid sequences identical to the synthetic peptides were expressed in *E. coli*. Peptides 1 through 5 (14-mer) spanned the Nt epitope-containing region (amino acids 29 to 84), peptides 6 through 9 (14-mer) spanned the Ct epitope-containing region (amino acids 942 to 985), peptides 10 through 15 (14-mer) spanned one Ct epitope-containing region (amino acids 1222 to 1287), and peptides 16 through 21 (15-mer) spanned the distal Ct epitope-containing region (amino acids 1333 to 1402). All peptides overlapped by four amino acids (Table 3).

Western immunoblotting and ELISA were performed to determine the recombinant gp200 peptide immunoreactivity, and the immunoreactivities of the synthetic peptides were evaluated by ELISA. Recombinant peptides 2, 5, 9, 15, and 20 exhibited strong immunoreactivity, and peptides 8, 11, 16, and 19 were weakly immunoreactive by Western blotting (Fig. 4B). Seven recombinant peptides, 2, 5, 8, 9, 15, 19, and 20, were strongly immunoreactive by ELISA and were not identified by BLASTp as having significant homology with *E. chaffeensis* gp200 or any other ehrlichial protein (Fig. 4C). Most synthetic peptides exhibited reduced immunoreactivity compared to the corresponding recombinant expressed fusion polypeptides, indicating a conformational dependence of these epitopes (Fig. 4C). A 14-mer positive-control peptide derived from *E. chaffeensis* p28-19 was included to demonstrate peptide binding and immunoreactivity and was strongly reactive with convalescent dog anti-*E. canis* antibodies.

Serine residues and antibody binding. Serine is a frequently occurring amino acid in immunoreactive ehrlichial proteins and epitopes and was the second most common ($n = 121$) amino acid present in gp200. Recombinant peptides 2 and 20 were strongly reactive by ELISA, and each contained a single serine residue. Thus, these peptides were chosen for site-directed mutagenesis to examine the contribution of serine residues in antibody binding. Site-directed serine mutants (sequences shown in Table 3) of peptides 2 and 20 were substantially less immunoreactive by ELISA than were the corresponding wild-type peptides (Fig. 5), demonstrating that these serine residues are important determinants of these two epitopes.

DISCUSSION

Antibodies are important for protection against *Ehrlichia* (7, 9, 10, 12, 31, 32), but the immunoprotective proteins and protective epitopes remain unknown. Many of the major immunoreactive proteins of *E. canis* and *E. chaffeensis* have been recently characterized, and most have serine-rich tandem repeats (5, 15, 16, 19, 34, 35). Furthermore, dominant antibody

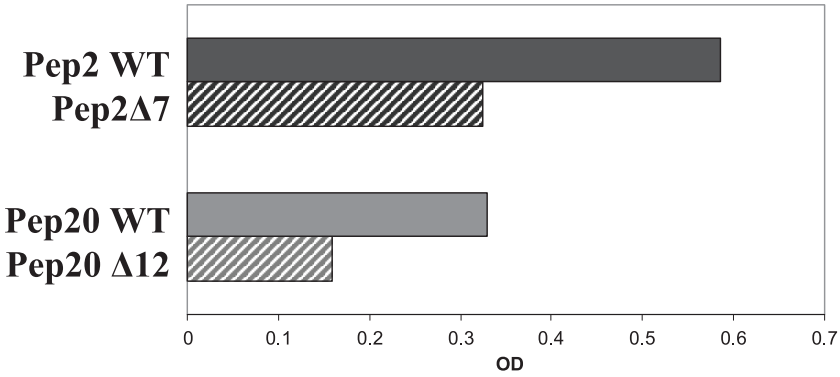

FIG. 5. Comparison of antibody reactivities as determined by ELISA between wild type (WT) and single serine-to-alanine mutant recombinant *E. canis* gp200 peptides 2 and 20 containing dominant epitopes. OD, optical density.

epitopes in these proteins have been mapped to acidic serine-threonine-rich regions and serine-rich tandem repeats in all of these proteins (5, 19, 33, 35). Thus, serine-rich acidic epitopes appear to be primary targets of the humoral immune responses against ehrlichiae and may be immunoprotective. In this study, five species-specific epitopes were identified primarily in terminal highly acidic domains of *E. canis* gp200.

The *E. canis* and *E. chaffeensis* gp200 orthologs have identical chromosomal locations and exhibit nucleic acid homology (~50%) but have less amino acid identity (~32%) (15). We previously reported that the N-terminal region (P43) (analogous to the Nt in this study) of gp200 was strongly recognized by antibody from *E. canis*-infected dogs and that antibodies directed at P43 were species specific (16). Ehrlichiae are known to have serologically cross-reactive antigens. The best-characterized examples are the major outer membrane proteins (p28/p30), map2, and other highly conserved immunoreactive proteins such as the chaperonin GroEL, disulfide oxidoreductase (Dsb), and ferric ion binding protein (Fbp) (1, 2, 6, 20, 24, 25, 29, 38). However, several of the epitopes characterized in the major immunoreactive proteins of *E. canis* and *E. chaffeensis* are molecularly distinct and elicit species-specific antibodies (5, 19). Consistent with our previous findings regarding the Nt epitopes (P43), the amino acid alignments of the mapped epitopes in gp200 (Nt, Ci, and Ct) identified no significant homology with *E. chaffeensis* gp200. Interestingly, all of the major immunoreactive protein orthologs (gp36/gp47, gp120/gp140, and gp19/VLPT) identified and characterized recently are antigenically distinct, further suggesting that cross-reactive antibodies generated between closely related *Ehrlichia* spp. are directed at more highly conserved proteins that are not necessarily considered to be major immunoreactive proteins.

The terminal epitope-containing domains exhibited high proportions of charged amino acids including glutamate and aspartate and the hydrophobic amino acid valine. The majority of the epitopes (except the Ci epitope [peptides 8 and 9]) were located within the first 100 amino acids or the last 200 amino acids of the protein, within these highly acidic terminal domains, flanking the ankyrin domain-containing region. The high proportion of acidic and polar amino acids observed in these terminal domains has been consistently observed in other characterized ehrlichial epitopes (5, 19). Furthermore, most of the characterized immunoreactive ehrlichial proteins (i.e., gp36, gp47, gp19, gp140, gp120, and gp200) are strongly acidic (pI ~4 to 5.4). The role of the acidic, polar, and hydrophobic amino acids, such as glutamate, serine, and valine, is not currently understood, but the consistent presence of these amino acids in ehrlichial epitopes suggests that they constitute an important motif that interacts strongly with the host immune response. The predominance of antibody response to acidic ehrlichial proteins, particularly those containing tandem repeats, has not been described in relation to any other pathogen and suggests that an important and unique ehrlichia-host interaction occurs that directs the immune response towards these proteins.

The genome of *E. canis* has a revealed a serine/threonine bias in proteins associated with host-pathogen interactions (14). The polar amino acid serine was the second most frequently occurring amino acid in *E. canis* gp200 (n = 121), and the largest proportions of serine and threonine residues (73 residues combined; 17) were identified in the Ct region of *E. canis* gp200. They are also highly represented in other major immunoreactive ehrlichial proteins (5, 19, 34, 35). Many of these proteins are associated with host-pathogen interactions (14, 34). Serine was present in all (peptides 2, 5, 8, 15, 19, and 20) but one (peptide 9) of the peptides that exhibited the strong antibody recognition by *E. canis*-infected dog sera. Mutations of single serine residues present in peptides 2 and 20 did substantially reduce (50%) the immunoreactivity of the peptides, suggesting that serine residues are critical for the binding of antibodies to these epitopes. Notably, the high frequency of serine residues in immunoreactive ehrlichial proteins including gp200 has not been reported for other bacteria, but the high frequency of these residues suggests an important role in the pathobiology of ehrlichiae. Furthermore, there appears to be a direct relationship between the host immune response and acidic, serine-rich proteins.

Antibody epitopes mapped in other ehrlichial proteins appear to be single epitopes located in acidic serine-rich tandem repeats (5, 19, 33–35). However, we found numerous molecularly distinct epitopes in gp200 that were not located in tandem repeats. The number of epitopes identified in gp200 suggests that it is an important target of the host immune response against *E. canis*. Epitopes that have been mapped in other ehrlichial proteins are present in tandem repeat-containing

proteins that are associated with host-pathogen interactions (27, 35). gp200 is translocated to the host cell nucleus (23), which has also been reported for AnkA, a similar ankyrin-containing protein found in *A. phagocytophilum* (26). Furthermore, AnkA has been proposed to be a type IV secreted virulence factor because of its potential ability to facilitate intracellular infection by activation of the host Abl-1 signaling pathway, recruitment of phosphatase SHP-1, and DNA binding (8, 13, 26). Although the role of antibodies in inhibiting the functional role of *E. canis* gp200 is unknown, inhibition of gp200 by the host immune response would likely result in an outcome beneficial to the host.

The immunoreactivity of the 21 synthetic peptides compared to the recombinant fusion peptides suggests that these epitopes have a conformational dependence, which is facilitated by fusion of the peptide to the thioredoxin fusion protein. Conformational dependence of antibody epitopes in *Anaplasma* and *Ehrlichia* spp. has been recognized in several proteins such as Fbp and map2 in *E. canis* and msp5 in *Anaplasma marginale* (2, 6, 13, 22). In this case, the strong antibody recognition of the synthetic *E. chaffeensis* p28-19 peptide demonstrated strong binding by synthetic peptides to the solid substrate used in this study. Therefore, the general lack of immunoreactivity of the synthetic peptides compared to the recombinant fusion gp200 peptides is most likely a result of improved solubility and appropriate epitope folding, resulting in increased antibody binding.

The *E. canis* and *E. chaffeensis* gp200s are the largest immunoreactive proteins (15, 17), and these unique ankyrin domain-containing proteins warrant further investigation. gp200-specific antibodies are elicited in dogs experimentally and naturally infected with *E. canis* (16, 17), and we have found that gp200 contains the largest number of distinct antibody epitopes reported in any ehrlichial immunoreactive protein. Additional studies are needed to determine the protection mediated by antibody against the *E. canis* gp200 epitopes to fully appreciate its potential as an immunoprotective antigen.

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