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Intrahaplotype and interhaplotype pairing of bovine leukocyte antigen DQA and DQB molecules generate functional DQ molecules important for priming CD4+ T-lymphocyte responses

Abstract Antigen-specific CD4+ T-lymphocyte responses are restricted by major histocompatibility complex class II molecules, which influence T-cell priming during infection. Human leukocyte antigen (HLA) and bovine leukocyte antigen (BoLA) *DRB3* and *DQ* genes are polymorphic, but unlike HLA, many BoLA haplotypes have duplicated *DQ* genes, and antibody-blocking studies indicated that BoLA-DQ molecules present various pathogen epitopes. Limited experimentation also suggested that BoLA-DQ molecules formed by interhaplotype pairing of A and B chains are functional. To compare antigen presentation by DR and DQ molecules and to definitively demonstrate functional BoLA-DQ molecules derived from interhaplotype pairing, different combinations of DR or DQ A and B proteins were expressed with CD80 in 293-F cells for use as antigen-presenting cells (APCs). This approach identified 11 unique restriction elements including five DR and six DQ pairs for antigen-specific CD4+ T-cell responses against tick-transmitted bovine hemoparasites *Anaplasma marginale* or *Babesia bovis*. Interhaplotype pairing of DQ A and B molecules was demonstrated. Testing of six expressed *DQA/B* pairs from an animal with duplicated DQ haplotypes (DH16A/DH22H) demonstrated that an interhaplotype pair, *DQA*2206/DQB*1301*, presented *A. marginale* peptide B. In DH22H and DH16A homozygous animals, *DQA*2206* was tightly linked with *DQB*1402*, and *DQA*22021* was linked with *DQB*1301*. APCs from these donors could not present peptide B, confirming that *DQA*2206/DQB*1301* encoded a functional interhaplotype pair. Functional BoLA-DQ molecules are generated by both intrahaplotype and interhaplotype pairing of A and B chains and play a similar role to BoLA-DR in priming helper T-cell responses to important pathogens.

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Introduction

The main function of major histocompatibility complex (MHC) class II molecules is to present processed pathogen-derived peptides to CD4+ T lymphocytes. MHC class II alleles are redundant and highly polymorphic, enhancing the repertoire of epitopes that an individual can recognize. Whereas mice express two class II proteins, I-E and I-A, humans express three (HLA-DR, HLA-DQ, and HLA-DP), and heterozygous individuals can therefore express at least four (mice) or six (humans) sets of class II gene products. Cattle express only two class II proteins, DR and DQ. However, in the majority of haplotypes expressed by Holstein cattle, bovine leukocyte antigen (BoLA)-DQ molecules are duplicated, permitting additional diversity through intrahaplotype and, potentially, interhaplotype pairing of DQA and DQB proteins, as reported for human DQA and DQB (Kwok et al. 1993). Interhaplotype pairing refers to pairing of *DQA* and *DQB* gene products encoded by different chromosomes. When DQ genes are duplicated, intrahaplotype pairing refers to pairing of *DQA* and *DQB* gene products encoded by the same chromosome, which can occur either within a *DQA/DQB* locus (referred to as "adjacent" pairing) or between the sets of duplicated gene loci ("nonadjacent" pairing) but within the same haplotype. Because the DRA chain in both humans and cattle is monomorphic, polymorphism in *DRB* genes is the only source of diversity in DR molecules. In cattle, only one *DRB* (*DRB3*) gene is known to be functional, whereas humans use *DRB1* predominantly, but *DRB3*, *DRB4*, and *DRB5* genes are functional in some haplotype groups (Schreuder et al. 2005). Unlike DRA, both HLA- and BoLA-DQA and DQB chains exhibit polymorphism, which would intuitively be used by the immune system to increase the antigenic epitope-binding repertoire (Lewin et al. 1999). However, for HLA-DQ, this does not appear to be the case. For reasons that are not understood, the great

majority of human peripheral blood derived T-cell clones is restricted by DR molecules (Mølvig et al. 1989; Endl et al. 1997), although HLA-DQ molecules can present peptides from different types of pathogens (Calvo-Calle et al. 1997; Agrewala and Wilkinson 1998; Kwok et al. 2000). The best studied role of HLA-DQ molecules is their association, either positive or negative, with immunologically mediated diseases including insulin-dependent diabetes mellitus (Abbas et al. 2000) and celiac disease (Sollid et al. 1989).

In cattle, *BoLA-DRB3* exon 2 has been extensively sequenced and most of the *DRB3* alleles in Holstein cattle are known, whereas the sequence information for *BoLA-DQ* genes and the understanding of the role of BoLA-DQ molecules in bovine immune system are limited. To date, 106 *DRB3*, 46 *DQA*, and 52 *DQB* alleles have been reported (http://www.projects.roslin.ac.uk/bola, http://www.ebi.ac.uk/ipd/mhc/bola). Since *BoLA-DRA* is monomorphic and *DRB3* is the only active *DRB* gene, it is likely that DQ molecules are as important as DR molecules for priming CD4$^+$ T cells specific for many different pathogens. Using monoclonal antibody (mAb)-blocking assays, Glass et al. (2000) demonstrated presentation by BoLA-DQ molecules of foot-and-mouth disease virus (FMDV) epitopes to CD4$^+$ T cells. We have similarly shown presentation of *Babesia bovis* and *Anaplasma marginale* epitopes to CD4$^+$ T cells by BoLA-DR and BoLA-DQ molecules (Brown et al. 2002, 2003; Norimine et al. 2002, 2004). However, in our studies, ambiguous results were obtained with mAb used to block proliferation of a T-cell clone (Norimine et al. 2002; W.C. Brown, unpublished observations), and the specificity (DR vs DQ) of one mAb reagent (IL-A21) was also reportedly uncertain (Fogg et al. 2001). Nevertheless, evidence for functional interhaplotype pairing of DQ molecules was presented in studies using T-cell clones specific for FMDV (Glass et al. 2000) or *A. marginale* major surface protein (MSP)-1a (Brown et al. 2002). In both studies, the pattern of peptide presentation by antigen-presenting cells (APCs) expressing certain combinations of haplotypes was indicative of interhaplotype pairing of DQA and DQB chains, but definitive proof of this was lacking (Glass et al. 2000; Brown et al. 2002).

The present study used a transfected cell line expressing BoLA-DQ or BoLA-DR A and B proteins to determine whether DQ and DR molecules present antigen to CD4$^+$ T cells with comparable frequency and to definitively demonstrate that interhaplotype pairing of BoLA-DQA and BoLA-DQB molecules results in the formation of functional DQ heterodimers. T-cell lines and clones specific for known epitopes of the bovine pathogens *B. bovis* and *A. marginale* were used. In addition, *BoLA-DQ* and *BoLA-DR* alleles were sequenced from the T-cell donors, and different gene pairs, together with bovine *CD80* or *CD86*, were expressed in a human embryonic kidney cell line and used as a source of APCs. Our results show that BoLA-DQ molecules function like BoLA-DR molecules to present pathogen-derived antigenic peptides and therefore differ from HLA-DQ molecules.

Materials and methods

Experimental cattle and their BoLA haplotypes

All of the protocols in this study were reviewed and approved by the Washington State University Institutional Animal Care and Use Committee. Brahman–Angus cross cow C97 was infected with the Mexico strain of *B. bovis* (Brown et al. 1991), Holstein steer 87 was immunized with *A. marginale* native MSP1, which is a heterodimer of MSP1a and MSP1b (Brown et al. 2001a), and Holstein steer 61 was immunized with *A. marginale* native MSP2 (Brown et al. 2001b) as previously described. Holstein steer 04B93 and cows 131, 132, and 201 were used as nonimmune controls. The *DRB3* alleles of animals C97, 61, 87, and 04B93 were determined by the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method as developed by van Eijk et al. (1992a). RFLP analysis was particularly important for confirming whether animals had homozygous BoLA haplotypes. Further, each PCR product from exon 2 of each *DRB3* gene was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced using the M13 forward and reverse primers. Sequencing was performed using the Prism Ready Reaction Dye Deoxy Terminator cycle-sequencing kit and analyzed with the ABI Prism 373 genetic analyzer (Applied Biosystems). For all animals, *DQA1* and *DQA2* alleles were determined by sequencing their exon 2 regions using recently published primer sets (Park et al. 2004). All *DQB1* and *DQB2* alleles were identified by sequencing the entire cDNAs using the primer sets listed in Table 1. The class II haplotypes of all cattle are shown in Table 2.

Antigens and synthetic peptides

Babesia bovis Mexico strain merozoite antigen enriched in parasite cell membranes (CM) was prepared from cultured, infected erythrocytes following homogenization with a French pressure cell and centrifugation as described (Brown et al. 1991). *A. marginale* Florida strain organisms were isolated from thawed, infected bovine erythrocytes by sonication and differential centrifugation as described (Palmer and McGuire 1984). Antigens were diluted in phosphate-buffered saline (PBS; pH 7.4) containing 25 μg/ml of the protease inhibitors antipain and E-64 (Boehringer Mannheim) and 300 μg/ml phenylmethylsulfonyl fluoride (Sigma) and stored at −20°C. All peptides were synthesized by Gerhardt Munske, Laboratory for Biotechnology and Bioanalysis I, Washington State University, Pullman. The peptides were diluted to 1 mg/ml in PBS and stored at ⁻20°C. The amino acid sequences of the peptides and their origin are listed in Table 3.

Table 1 Primer sets used for *BoLA-class II*, *CD80*, and *CD86* constructs

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession[b]
DRA	CGAGACACCGAAGAAGAAAATGGC	TCACAGAGGCCCTCGGCGTTC	D37956
DRB3	GGCATGGTGTGCCTGTATTTCTCTG	TCAGCTCAGGAGCCCTGTTGG	Y18246
DQA1[a]	AGGATGATCCTGAACAGAGCCCTGA	TCACAACGGCCCCTGGTGTCT	Y07898
	AGGATGGTCCTGAACAGAGCCCTGA	TCACAACGGCCCCTGGTGTCT	Y07819
DQA2	AGGATGGTCCTGAACAGAGCTCTGA	CTAGGGTGCAACTCACAAGGGA	Y07820
DQB1	ATTATGTCTGGGATGGTGGCTCTGT	CAGAAGAGCAAAATCCAGTCTCCAG	Y18203
DQB2[a]	ATTATGTTTGGGATGGTGGCTCTGC	GCAGCATCACAGCCAGATCCGT	Y18202
	ATTATGTCTGGGATGGTGGCTTTGC	TATCCTCAGGAGTCAGCGCA	Y18201
CD80	GCCATGGGTCACACAATGAAGT	TCATGGAGACTGAGAGCAACTTCC	Y09950
CD86	ACAGCAGAAATAACGAAAATGCG	CATGGCGTTTACTCTTTAATTACA	AJ291475

[a] These primer sets were used interchangeably
[b] GenBank accession number

Transfection of 293-F cells with MHC class II, CD80, and CD86 pCR3.1 constructs

Total RNA was obtained from peripheral blood mononuclear cells (PBMCs) of each animal using Trizol Reagent (Invitrogen) and reverse-transcribed to cDNA in a 50-µl volume by using oligo $(dT)_{16}$ (Perkin Elmer) according to the manufacturer's instructions. Amplification of full-length cDNA for *DRA*, *DRB3*, *DQA*, *DQB*, *CD80*, and *CD86* was performed by PCR using the primer sets listed in Table 1. The PCR parameters were 94°C 10 min, 35 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 2 min, with an extension of 72°C for 10 min, and finally 4°C. Each PCR

product was cloned into the eukaryotic expression vector pCR3.1 (Invitrogen) using T4 ligase (Invitrogen). The ligation was performed overnight at 15°C. The sequence and direction of each insert was confirmed by sequencing in both direction using T7 forward and BGHR primers (Invitrogen). For use in transfection, 90% confluent human embryonic kidney 293-F cells (Invitrogen) were cultured in a six-well plate in Dulbecco's Minimal Essential Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Atlanta Biologicals) and penicillin–streptomycin. Prior to transfection, the cells were washed once with DMEM (without FCS and antibiotics), and 800 µl of DMEM (without FCS and antibiotics) was added to each well. A plasmid DNA mixture consisting of

Table 2 *DRB3*, *DQA*, and *DQB* alleles identified in the cattle used in this study

Animal[a]	Haplotype	DRB3		DQA	DQB	Note
		RFLP	Allele	Allele	Allele	
C97	NA[b]	34	*3001	*0301	*0402	Heterozygous[e]
		ND[c]	*4501	*C97[d]	*C97[d]	
61	DH08A	8	*1201	*12011	*10051	Homozygous
				*2201	*1201	
87	DH16A	16	*1501	*10011	*10021	Heterozygous
				*22021	*1301	
	DH22H	22	*1101	*10011	*10021	
				*2206	*1402	
04B93	DH16A	16	*1501	*10011	*10021	Homozygous
				*22021	*1301	
131	DH16A	16	*1501	*10011	*10021	Homozygous
				*22021	*1301	
132	DH22H	22	*1101	*10011	*10021	Homozygous
				*2206	*1402	
201	DH22H	22	*1101	*10011	*10021	Homozygous
				*2206	*1402	

[a] C97 is a Brahman–Angus cow infected with *B. bovis*; 61 is a Holstein–Friesian steer immunized with native *A. marginale* MSP2; 87 is a Holstein–Friesian steer immunized with native *A. marginale* MSP1; 04B93, 131, 132, and 201 are naive Holstein–Friesian steers
[b] The haplotype designation for C97 is not available
[c] *DRB3* RFLP was not determined
[d] Because these allele names are not available, the name C97 has been temporarily used for the *DQA* (*DQA*C97*) and *DQB* (*DQB*C97*) alleles. The mRNA sequences were submitted to GenBank (accession numbers AY730727 and AY730728, respectively)
[e] Linkages are not known

Table 3 *B. bovis* and *A. marginale* peptides used in this study

Protein[a]	Name	Amino acid position[b]	Sequence	Reference
RAP-1	P3	174–203	NNNVVHEEGTTDVEYLVNKVLYMATMNYKT	Norimine et al. 2002
	P9	294–316	VEAPWYKRWIKKFRDFFSKNVTQ	Norimine et al. 2002
	CT-P2	386–408	PTKEFFREAPQATKHFLDENIGQ	Norimine et al. 2002
Hsp20	P1	11–40	DQEVIIDEQTGLPIKSHDYSEKPSVIYKPS	Norimine et al. 2004
MSP1a	Peptide B	29–203[c]	ADSSSAGGQQQESSVSSQSDQASTSSQLG	Allred et al. 1990; Brown et al. 2002
	F2-5B	243–258	ARSVLETLAGHVDALG	Brown et al. 2002
	F3-3	290–319	SDAADKFRVMMFGGAPAGQEKTAEPEHEAA	Brown et al. 2002
	F3-5	330–359	VHGKVVDAVDRAKEAAKQAYAGVRKRYVAK	Brown et al. 2002
MSP2	P25	272–291	VAGAFARAVEGAEVIEVRAI	Brown et al. 2001a, 2004
	P12-AM5	328–341	DGHINPKFAYRVKA	Brown et al. 2001a
	P16-7	394–409	NFAYFGGELGVRFAF	Brown et al. 2001a, 2004

[a]RAP-1 and Hsp20 proteins are derived from *B. bovis* (Mexico strain), and MSP-1a and MSP2 proteins are derived from *A. marginale* (Florida strain)

[b]Relative amino acid position of this epitope within the indicated protein

[c]Peptide B is tandemly repeated seven times in MSP1a of the *A. marginale* Florida strain (Allred et al. 1990)

2 µg of each plasmid DNA was added to 100 µl of DMEM (without FCS and antibiotics), mixed with 6 µl of Plus Lipofectamine (Invitrogen), and incubated at room temperature for 15 min. The DNA mixture was then combined with a Lipofectamine mixture consisting of 100 µl DMEM (without FCS and antibiotics) and 4 µl of Lipofectamine and incubated at room temperature for 15 min. The DNA–Lipofectamine complex was added to the 293-F cells. After incubation for 3 h at 37°C, 1 ml of DMEM with 4% FCS (without antibiotics) was added, and the cells were cultured for 2 days. Medium was removed, complete RPMI-1640 medium (Brown et al. 1991) containing 50 µg/ml Mitomycin C (Sigma) was added, and the cells were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The transfected cells were harvested by repetitive pipetting, washed three times with complete RPMI-1640 medium, and used as APCs.

Flow cytometric analysis of 293-F cells transfected with BoLA-class II, CD80, and CD86

Expression of BoLA-class II and costimulatory molecules on transfected 293-F cells was verified by flow cytometry using bovine DR-specific mAb IL-A21, DQ-specific mAbs TH22A and CC158, CD80-specific mAb IL-A159, and CD86-specific mAb IL-A190. mAb TH22A was purchased from the Washington State University Monoclonal Antibody Center. mAbs CC158, IL-A159, and IL-A190 were kindly provided by Chris Howard, Institute of Animal Health, Compton, UK, and Niall MacHugh, University of Edinburgh, UK. mAbs IL-A21, IL-A159, and IL-A190 were originally obtained from the former International Laboratory for Research on Animal Diseases (current International Livestock Research Institute), Nairobi, Kenya. For the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat antimouse immunoglobulins (IgA, IgG, IgM) (CAPPEL) was used. To confirm coexpression of CD80 and DR or DQ, the mixture of antibovine CD80 mAb IL-A159 (IgG1) and either anti-BoLA-DR mAb IL-A21

(IgG2a) or anti-BoLA-DQ mAb CC158 (IgG2a) was used for the primary antibody (15 µg/ml each mAb). For the secondary antibody, the mixture of FITC-conjugated goat antimouse IgG2a and R-phycoerythrin (R-PE)-conjugated goat antimouse IgG1 (CALTAG Laboratories) was used.

T-cell proliferation assays

To establish short-term T-cell lines from animals 61 and 87, PBMCs were initially depleted of $CD8^+$ and γδ T lymphocytes using antibody and complement lysis. Briefly, PBMCs were incubated for 30 min at 4°C with anti-CD8 mAb 7C2B and anti-TCR γ chain mAb CACT61A purchased from the Washington State University Monoclonal Antibody Center and diluted to 15 µg/ml in complete RPMI-1640 medium. The cells were washed once and incubated for 30 min at 37°C with rabbit complement (Sigma) diluted 1:16 in complete medium and washed twice. Viable cells were purified using a Histopaque 1083 (Sigma) gradient. Subsequently, 4×10^6 cells were cultured in 24-well plates (Costar) in 1.5 ml of complete medium with 10 µg/ml homogenate prepared from the Florida strain of *A. marginale* (Brown et al. 1998, 2001b). After 7 days, lymphocytes were washed in complete medium, and 7×10^5 cells/well were cultured for 7 days with 2×10^6 irradiated autologous PBMCs without antigen. Short-term T-cell lines from animal C97 were similarly established without $CD8^+$ and γδ T-lymphocyte depletion and using 10 µg/ml *B. bovis* CM antigen to stimulate the T cells. $CD4^+$ T-cell clones specific for *A. marginale* MSP1a, MSP2, or *B. bovis* small heat shock protein Hsp20 were described elsewhere (Brown et al. 2002, 2004; Norimine et al. 2004). Proliferation assays were performed for 3–4 days in duplicate or triplicate wells of round-bottomed 96-well plates (Costar) at 37°C in a humidified atmosphere of 5% CO_2 in air. Briefly, 3×10^4 T cells and 2×10^5 autologous APCs were cultured with 0.1–10 µg/ml of antigen or peptide. The cells were radiolabeled during the last 18 h of culture with ^{3}H-thymidine, and the results are reported as mean cpm of

replicate cultures. In one experiment, APCs were prepared from nonautologous donors as described in the text. When transfected 293-F cells were used as APCs, 293-F cells were treated with 50 μg/ml Mitomycin C, plated in 96-well U-bottomed plates (5×10^4 cells/well), loaded with 0.1–10 μg/ml of peptide in triplicate wells in a total volume of 100 μl complete RPMI-1640 medium, and incubated for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The plates were washed 2–3 times with complete RPMI-1640 medium by centrifugation at $900 \times g$, and $CD4^+$ T cells derived from either short-term cell lines or $CD4^+$ T-cell clones (3×10^4 cells/well) were then added in a total volume of 100 μl complete RPMI-1640 medium. Cells were cultured for 3–4 days, radiolabeled, harvested, and counted.

Statistical significance of antigen-specific T-cell proliferation to peptide presented by different irradiated PBMCs as a source of APCs, untransfected 293-F cells, or 293-F cells transfected with different MHC class II A and B chains was evaluated by the Student's two-tailed t test. P values less than 0.05 are considered significant.

Results

BoLA-DR and BoLA-DQ alleles and their linkages in the experimental animals

The *BoLA* class II alleles identified are based on BoLA nomenclature web site (http://www.projects.roslin.ac.uk/bola, http://www.ebi.ac.uk/ipd/mhc/bola) and are shown in Table 2. Because the names of one *DQA* and one *DQB* allele from Brahman–Angus cross cow C97 were not avail-

able on the web site, the *DQA* allele (accession number AY730727) and *DQB* allele (accession number AY730728) have been designated *DQA*C97* and *DQB*C97* alleles, respectively. Animal 87 had heterozygous BoLA haplotypes (DH16A/DH22H), each of which contained duplicated DQ pairs. The *DR-DQ* linkages were confirmed by sequencing *DR* and *DQ* genes from animals 04B93 and 131 (DH16A homozygous) and from animals 132 and 201 (DH22H homozygous). As shown in Table 2, the *DQA* 2206* and *DQB*1402* alleles are linked with the *DRB3* 1101* allele (RFLP 22) in haplotype DH22H, while the *DQA* 22021* and *DQB* 1301* alleles are linked with the *DRB*1501* allele (RFLP 16) in haplotype DH16A. The pair of *DQA*10011* and *DQB*10021* alleles was consistently observed in both DH22H and DH16A haplotypes. Extensive sequencing and *DRB3* RFLP analyses confirmed these homozygosities and linkages, and the linkages were consistent with results from sequencing *DRB3* exon 2 from other Holstein cattle (data not shown). Animal C97 has unique heterozygous BoLA haplotypes (both not defined) with nonduplicated *DQ* alleles. The linkage between *DR* and *DQ* genes in this animal is unknown since no animals sharing these *DR* or *DQ* genes were available for analysis.

Flow cytometric analysis of DR, DQ, CD80, and CD86 expression on 293-F cells

Bovine *CD80* and *CD86* cDNAs were also cloned into the expression vector pCR3.1 and examined for their expression on transfected 293-F cells by flow cytometric analysis using mAb IL-A159 (anti-CD80) and IL-A190 (anti-CD86).

Table 4 Expression of BoLA-DR and DQ molecules on 293-F cells

Animal		BoLA-class II molecule		Expression[a]	Type of pairing[b]
		A chain	B chain		
C97 (heterozygous, nonduplicated DQ)	*DR*	*0101	*4501	+	
			*3001	+	
	DQ	*0301	*0402	+	Unknown
			*C97	+	
		*C97	*0402	+	Unknown
			*C97	+	
61 (homozygous, duplicated DQ)	*DR*	*0101	*1201	+	
	DQ	*12011	*10051	+	Intrahaplotype (adjacent)
			*1201	−	Intrahaplotype (nonadjacent)
		*2201	*10051	−	Intrahaplotype (nonadjacent)
			*1201	+	Intrahaplotype (adjacent)
87 (heterozygous, duplicated DQ)	*DR*	*0101	*1101	+	
			*1501	+	
	DQ	*10011	*10021	+	Intrahaplotype (adjacent)
			*1301	−	Intrahaplotype (nonadjacent)
			*1402	−	Intrahaplotype (nonadjacent)
		*22021	*10021	+	Intrahaplotype (nonadjacent)
			*1301	+	Intrahaplotype (adjacent)
			*1402	+	Interhaplotype
		*2206	*10021	+	Intrahaplotype (nonadjacent)
			*1301	+	Interhaplotype
			*1402	+	Intrahaplotype (adjacent)

[a] 293-F cells were transfected with a single set of *BoLA-DR* or *BoLA-DQ* gene and stained with mAbs IL-A21 (anti-DR) and CC158 (anti-DQ), and class II expression was determined by flow cytometry
[b] Intrahaplotype pairing of DQA and DQB chains is referred to as "adjacent" (within a locus) or "nonadjacent" (between loci) of duplicated *DQA and DQB* genes

Both proteins were expressed at similar levels, and no cross-reactivities between the mAbs were observed. Co-expression of CD80 and BoLA-DR or BoLA-DQ molecules on 293-F cells was confirmed by double staining. The level of MHC class II expression varied among combinations of A and B chains, ranging from approximately 40 to 80% (data not shown). Class II expression was never detected when 293-F cells were transfected with a single *A* or *B* chain pCR3.1 construct (data not shown). Table 4 summarizes the expression profiles of all possible DRA/B and DQA/B pairs in transfected 293-F cells. All *DRA/B* pairs were expressed, while expression of four *DQA/ B* combinations, *DQA*12011/DQB*1201* and *DQA*2201/ DQB* 10051* (animal 61) and *DQA*10011/DQB*1301* and *DQA* 10011/DQB*1402* (animal 87) was not detected. The *DQA*22021* and *DQA*2206* alleles were expressed in combination with all three *DQB* alleles tested, indicating that these alleles could form intra- and interhaplotype DQ molecules. The *DQA*10011* allele was expressed only with the *DQB*10021* allele, which was tightly linked. Although there are several conflicting reports regarding the specificity of IL-A21 (anti-DR) and TH22A (anti-DQ) mAbs (Fogg et al. 2001; Davis et al. 1987; Davies et al. 1994; Bissumbhar et al. 1994; Fraser et al. 1996), we did not observe any cross-reactivity with these reagents. The specificity of anti-DQ mAb CC158 was consistent with previous reports (Howard et al. 1997; Russell et al. 2000).

Presentation of antigen by BoLA-DR and BoLA-DQ transfected cells requires coexpression of CD80 or CD86

To determine if the MHC class II molecules expressed on 293-F cells were functional, T-cell proliferation assays were performed. Previous studies had identified numerous epitopes within *B. bovis* rhoptry-associated protein 1 (RAP-1) (Norimine et al. 2002, 2003), including CD4^{+} T-cell epitopes P3 (aa 174–203), P9 (aa 294–316), and CT-P2 (aa 386–408) using lymphocytes from animal C97 infected with *B. bovis* (Norimine et al. 2002). Since CD4^{+} T-lymphocyte responses against peptides P3, P9, and CT-P2 were apparently restricted by DR molecules as determined by mAb-blocking assays (Norimine et al. 2002), we examined products of *DRA/DRB3*3001* and *DRA/DRB3* 4501* derived from animal C97 for their ability to present antigen. As shown in Fig. 1, CD4^{+} T-lymphocyte responses against peptides P3, P9, and CT-P2 were restricted by *DRA/ DRB3*3001*-encoded (for P3 and P9) and *DRA/DRB3* 4501*-endoded (for CT-P2) class II molecules. Importantly, T-cell proliferation was not observed in the absence of CD80 or CD86 expression, indicating that expression of a B7 molecule was required in this system. Because the efficiency of costimulation was comparable between CD80 and CD86, CD80 was used in subsequent MHC class II transfection experiments.
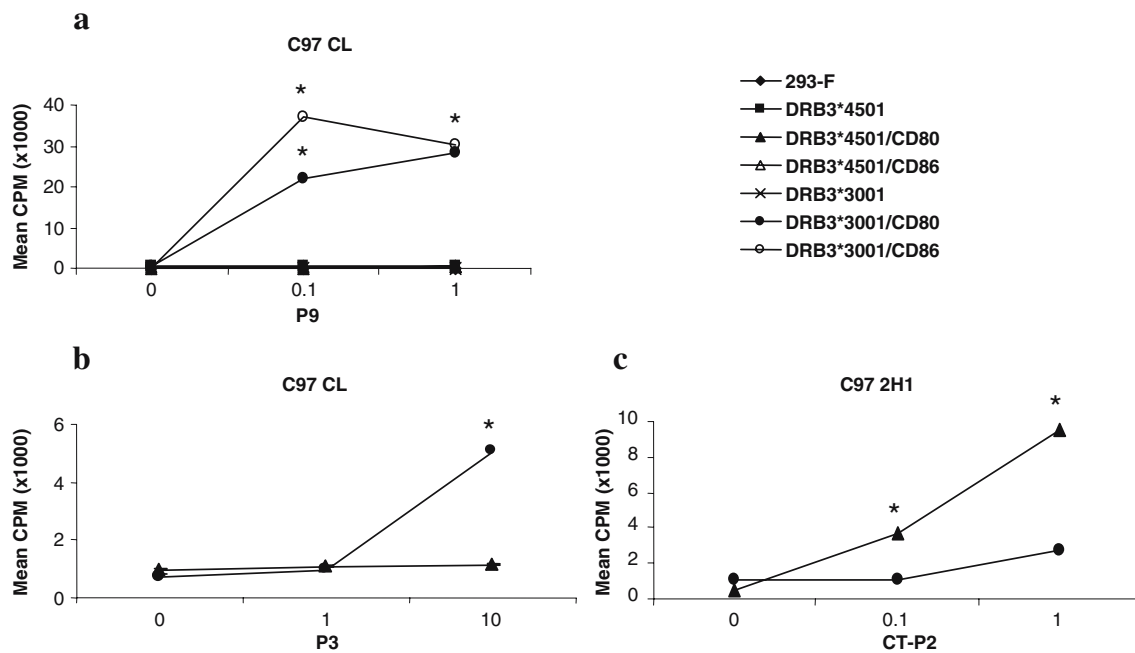

Fig. 1 Peptide-specific T-cell proliferation using *DR*-transfectants as APCs requires coexpression of either CD80 or CD86. 293-F cells were transfected with a single set of *DR* or *DQ A* and *B* chain alleles from animal C97 with or without the *CD80* or *CD86* pCR3.1 construct. T cells from short-term cell lines (C97 CL) were cultured with the indicated transfected cells loaded with the following peptides: RAP-1 peptide P9 (**a**), RAP-1 peptide P3 (**b**), and RAP-1 peptide CT-P2 (**c**). Results are presented as the mean cpm of triplicate or duplicate cultures. Responses significantly higher than responses to peptide in the presence of nontransfected 293-F cells are indicated by an *asterisk* (*P*<0.05) (**a**). Responses significantly higher than responses to peptide in the presence of 293-F cells expressing the other DRB3 allele are indicated by an *asterisk* (*P*<0.05) (**b** and **c**)

Presentation of *A. marginale* MSP2 epitopes by DR and DQ molecules

Anaplasma marginale MSP2-immunized animal 61 (Brown et al. 2001b) has a homozygous MHC haplotype (DH8A/DH8A) as shown in Table 2. The DH8A haplotype contains duplicated *DQ* alleles, so there are four potential DQ A and B pairs. However, only *DQA*12011/DQB*10051* and *DQA*2201/DQB*1201* allelic pairs were expressed on transfected 293-F cells (Table 4). This indicated that animal 61 expressed three MHC class II molecules, DRA/DRB3*1201, DQA12011/DQB*10051, and DQA*2201/DQB*1201. We determined if these MHC class II molecules were functional using three different MSP2 peptides previously shown to stimulate Th cells (Brown et al. 2001b, 2004; and W.C. Brown, unpublished data). Peptides P16-7, P25, and P12-AM5 from the conserved carboxy region of MSP2 were presented to CD4$^+$ T cells by DRA/DRB3*1201, DQA*12011/DQB*10051, and DQA*2201/DQB*1201 molecules, respectively (Fig. 2), confirming that all three MHC class II molecules were functional.

Evidence that heterozygous BoLA haplotypes generate functional interhaplotype DQ molecules

Previous mAb-blocking studies suggested that the *B. bovis* Hsp20 peptide P1 (aa 11–40), recognized by cow C97, was DQ-restricted (Norimine et al. 2004). To verify this and to determine the DQ restriction element, transfected 293-F cells were pulsed with peptide P1 and tested for stimulation of Hsp20-specific CD4$^+$ T-cell clone 3B11. Interestingly, this clone, which was shown to be derived from a single cell by sequencing the TCR α and β chains (Norimine et al. 2004), responded to peptide P1 presented by the products of two DQ allelic pairs, *DQA*0301/DQB*0402* and *DQA*C97/DQB*0402* (Fig. 3). This result was also obtained using CD4$^+$ T-cell clone 3G5 (Norimine et al. 2004) specific for the same epitope (data not shown). Because cow C97 does not have a duplicated haplotype (Table 2), this finding indicates that the *DQB*0402* allelic product paired with two different *DQA* allelic products, one provided from each haplotype (Tables 2 and 4), providing evidence for both intra- and interhaplotype pairing in the generation of functional DQA/B heterodimers.

Numerous epitopes on *A. marginale* MSP1a are recognized by CD4$^+$ T cells from MSP1-immunized cattle expressing DH16A and/or DH22H haplotypes (Brown et al. 2002). One of these, the N-terminal-repeated, 29-amino-acid-sequence peptide B, appeared to be restricted by interhaplotype-paired DQA and DQB molecules. Presentation of this epitope to T cells from heterozygous DH16A/DH22H animal 87 was blocked by DQ-specific mAb. Furthermore, this epitope was presented to CD4$^+$ T-cell clones only by autologous APCs or APCs from a donor animal expressing the identical heterozygous BoLA haplotype DH16A/DH22H, but not by APCs from cattle with
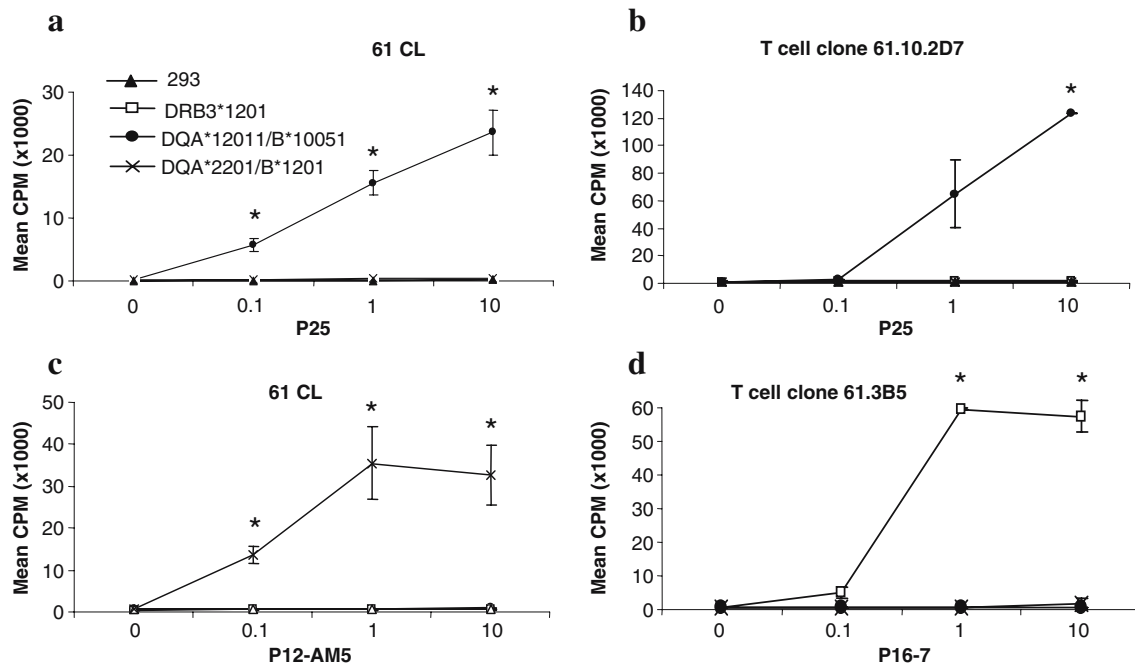

Fig. 2 Presentation of *A. marginale* MSP2 peptides by DR and DQ molecules. 293-F cells were transfected with *CD80* and *DRA/DRB3*1201*, *DQA*12011/DQB*10051*, or *DQA*2201/DQB*1201* and tested for presentation of loaded peptide. Short-term T–cell lines were stimulated with 293-F cells loaded with either 0.1–10 μg/ml peptide P25 (**a**) or peptide P12-AM5 (**c**), and CD4$^+$ T–cell clones 61.10.2D7 (**b**) and 61.3B5 (**d**) were stimulated with 293-F cells loaded with 0.01–10 μg/ml peptide P25 or 16-7, respectively. Results are presented as the mean CPM of triplicate cultures. Responses significantly higher than responses to peptide in the presence of nontransfected 293-F cells or 293-F cells expressing different class II alleles are indicated with an *asterisk* (*P*<0.05)
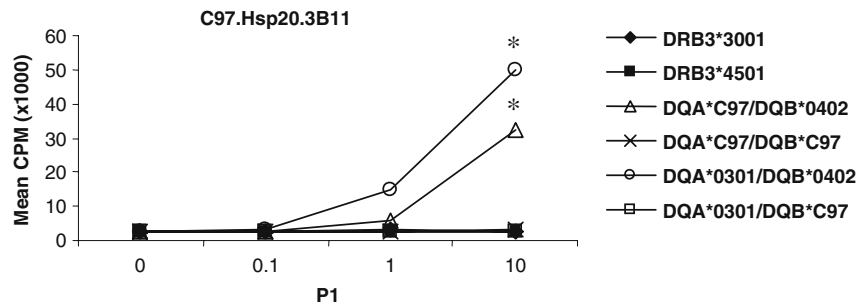
Fig. 3 Two DQA/B pairs present *B. bovis* Hsp20 peptide P1 to CD4$^+$ T-cell clone 3B11. *B. bovis* Hsp20-specific CD4$^+$ T-cell clone 3B11 was assayed with 293-F cells transfected with the indicated *DRB3* and *DQ* alleles and *CD80* and loaded with 0.1–10 μg/ml peptide P1.

Responses significantly higher than responses to peptide in the presence of 293-F cells transfected the other MHC class II allelic pairs are indicated by an *asterisk* (*P*<0.05)

one of the two sets of parental haplotypes (Brown et al. 2002). To confirm the presentation of peptide B by an interhaplotype DQ molecule, we first examined whether MSP1a peptide B-specific T cells from animal 87 could recognize the peptide presented by APCs from animals with homozygous DH16A (animals 04B93 and 132) or DH22H (animals 132 and 201) haplotypes (Table 2). Whereas autologous APCs presented peptide B, APCs from BoLA-homozygous donors expressing either DH16A/DH16A or DH22H/DH22H haplotypes were unable to present peptide B to a CD4$^+$ T-cell line derived from animal 87 expressing the DH16A/DH22A haplotype (Fig. 4a). These results support the previous observation that the presentation of MSP1a peptide B requires the heterozygous BoLA haplotype DH16A/DH22H, which would be necessary for generation of interhaplotype MHC class II molecules. In contrast, MSP1a peptide F3-5 (aa 330–359), also previously identified as a T-cell epitope recognized by animal 87 (Brown et al. 2002), was presented by APCs derived from DH22H-homozygous but not DH16A- homozygous animals (Fig. 4b).

To verify the use of interhaplotype-paired DQ molecules in the presentation of MSP1a peptide B, 293-F cells transfected with different *BoLA DQA/B* pairs were used to present the peptide. The intrahaplotype pair, *DQA*22021/ DQB*1301*, was not tested since expression of this pair of alleles was not detected when the initial experiments were performed. As hypothesized, MSP1a peptide B was clearly presented by the interhaplotype pair encoded by *DQA* 2206/DQB*1301* (Fig. 5a). We also confirmed that the intrahaplotype pair encoded by *DQA*2206/DQB*1402* within haplotype DH22H was functional for presenting a different peptide, F3-5 (Fig. 5b). These results indicate that, as demonstrated in vitro (Table 4), in vivo pairing of the *DQA*2206* allelic product with either *DQB*1301* or *DQB* 1402* allelic product resulted in the formation of different functional DQA/B heterodimers that primed CD4$^+$ T cells against two different MSP1a epitopes following immunization with MSP1.

Additional MSP1a-derived peptides, F2-5B (aa 243–258) and F3-3 (aa 290–319), also previously shown to contain CD4$^+$ T-cell epitopes (Brown et al. 2002), were included in the T-cell proliferation assays with MHC class II transfectants. *DQA*10011/DQB*10021* was identified as encoding the restriction element for CD4$^+$ T lymphocytes specific for MSP1a peptide F3-3 (Fig. 5c). Because this encoded DQA/B pair is present in both DH16A and DH22H haplotypes (Tables 2 and 4), animal 87 (DH16A/DH22H)
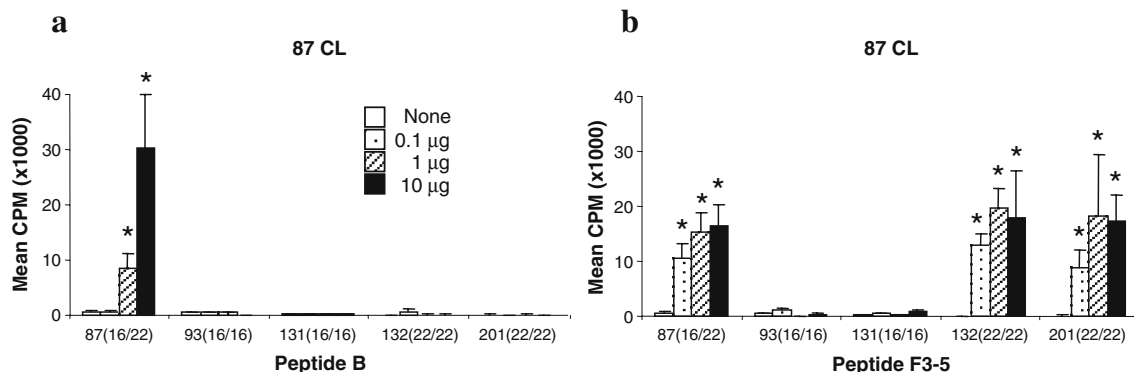

Fig. 4 Presentation of *A. marginale* MSP1a peptides B and F3-5 by APCs from *BoLA*-homozygous animals. T cells from a short-term cell line (87 CL) were cultured with 0.1–10 μg/ml peptide B (**a**) or peptide F3-5 (**b**) with autologous APCs or APCs derived from the indicated donors expressing homozygous DH16A or DH22H haplotypes. Results are presented as the mean cpm of triplicate cultures +1 SD. Responses significantly higher than responses without peptide are indicated by an *asterisk* (*P*<0.05)

a

b

c

d
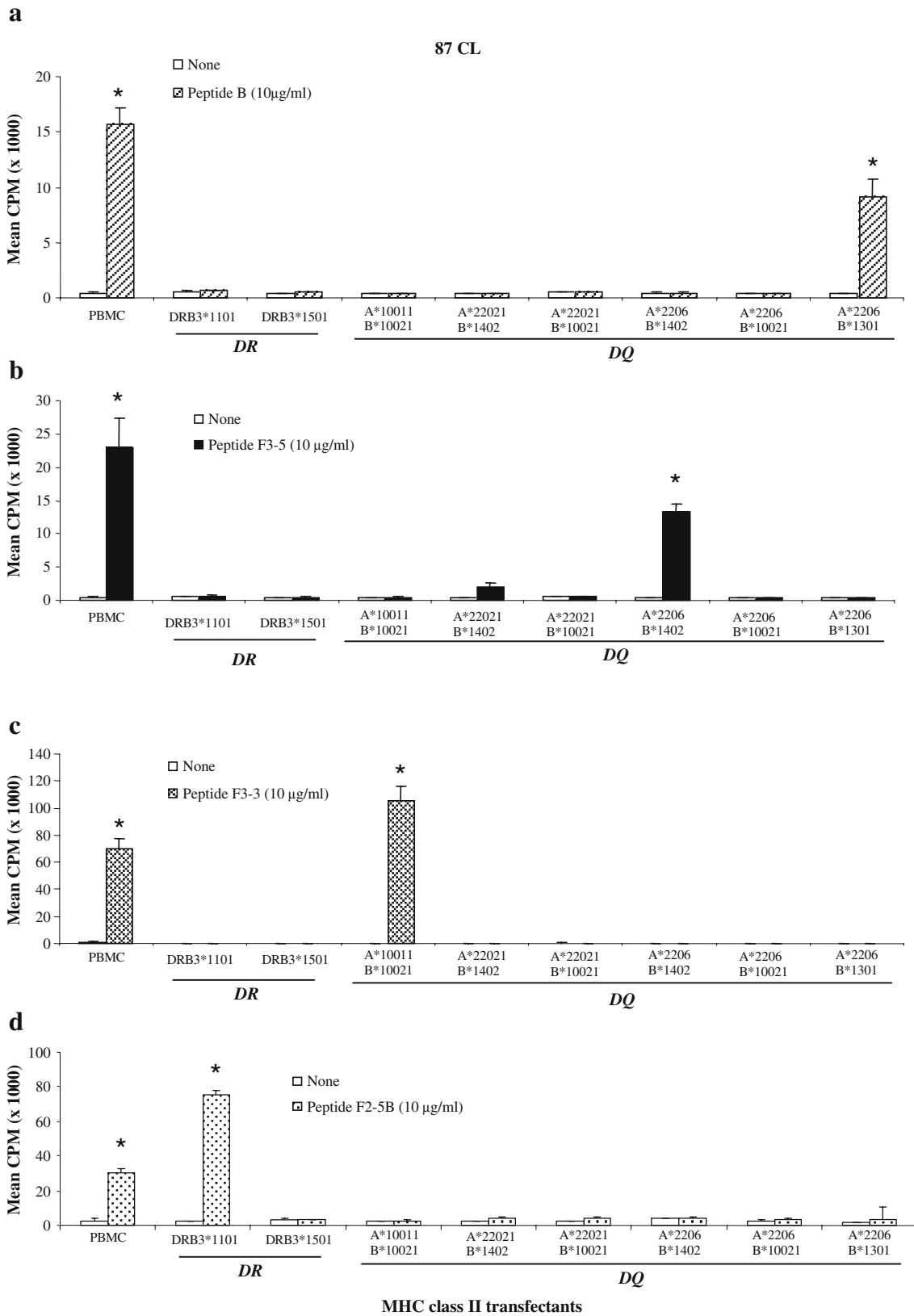
MHC class II transfectants

Fig. 5 Identification of BoLA-class II restriction elements for MSP1a epitopes using *DR*- and *DQ*-transfectants. T cells from a short-term T-cell line (87 CL) were cultured for 3 days with either irradiated, autologous APCs plus 10 μg/ml of the indicated peptide or with the indicated *DR*- or *DQ*- and *CD80*-transfected 293-F cells loaded with 10 μg/ml of *A. marginale* MSP1a peptide B (**a**), peptide F3-5 (**b**), peptide F3-3 (**c**), or peptide F2-5B (**d**). Results are presented as the mean cpm of triplicate cultures +1 SD. Responses significantly higher than responses without peptide are indicated by an *asterisk* (*P*<0.005)

Table 5 CD4^{+} T-cell epitopes examined and their BoLA-class II restriction elements

Animal	Peptide	BoLA-class II restriction element	T cells[a]
C97	RAP-1 P3	*DRA/DRB3*3001*	Line/Clone
	RAP-1 P9	*DRA/DRB3*3001*	Line
	RAP-1 CT-P2	*DRA/DRB3*4501*	Line/Clone
	Hsp20 P1	*DQA*0301/DQB*0402* and *DQA*C97/DQB*0402*	Line/Clone
87	MSP1a Peptide B	*DQA*2206/DQB*1301*	Line
	MSP1a F3-3	*DQA*10011/DQB*10021*	Line
	MSP1a F3-5	*DQA*2206/DQB*1402*	Line
	MSP1a F2-5B	*DRA/DRB3*1101*	Line/Clone
61	MSP2 P25	*DQA*12011/DQB*10051*	Line/Clone
	MSP2 P12-AM5	*DQA*2201/DQB*1201*	Line
	MSP2 P16-7	*DRA/DRB3*1201*	Clone

[a] T cells used to assay the peptides were either short-term T-cell lines, CD4^{+} T-cell clones, or both

might have an increased frequency of memory CD4^{+} T lymphocytes restricted by DQ molecule. The relatively high level of T-cell proliferation to peptide F3-3 supports this possibility. Finally, *DRB3*1101* was identified as encoding the restriction element for MSP1a peptide F2-5B (Fig. 5d), which again is consistent with previous studies showing that a DRB3 product linked to RFLP 22 presented this peptide to CD4^{+} T-lymphocyte clones (Brown et al. 2002).

Comparison of DR vs DQ presentation of epitopes for the proteins studied

The results of antigen presentation by BoLA-DQ or BoLA-DR are summarized in Table 5. A total of 11 unique restriction elements were identified in these studies; four encoded by DR-AB allelic pairs and seven encoded by DQ-AB allelic pairs. Furthermore, five different peptides were presented by DR molecules, and six different peptides were presented by DQ molecules. These results indicate that, at least among the proteins studied, comparable numbers of epitopes were presented by BoLA-DR and BoLA-DQ.

Discussion

This study has definitively shown functional BoLA-DQA/B heterodimers formed by interhaplotype pairing of DQA and DQB molecules. Interhaplotype pairing of DQA and DQB chains, together with duplication of *BoLA-DQ* genes, intrahaplotype DQA and DQB chain pairing, and polymorphism in *BoLA-DRB3* and *BoLA-DQ* genes, serves to increase the complexity of restriction element usage in cattle, which may influence the immunological outcome during infection (Lewin et al. 1999; Ellis and Ballingall 1999; Glass et al. 2000; Park et al. 2004;). In mice, mixed-haplotype H-2 molecules play an important role in causing autoimmune symptoms or a dominant immune response to hen egg lysozyme (HEL) (Moreno et al. 1990; Gotoh et al. 1993; Nygard et al. 1993). Strikingly, in (H-2^k×H-2^b)F$_1$ mice, 86% of T-cell hybridomas specific for HEL were restricted by mixed haplotypes, either I-Aα^{k}β^b or I-Aα^{b}β^k (Moreno et al. 1990). This observation is probably relevant to antigen presentation by HLA- and BoLA-DQ molecules because most humans and cattle have heterozygous haplotypes and both DQA and DQB chains are highly polymorphic, similar to mouse I-A molecules.

In cattle, duplication of DQ molecules provides an increased opportunity for inter- and intrahaplotype pairing of A and B chains to form functional DQ heterodimers. We identified the product of the interhaplotype DQ molecule, DQA*2206/DQB*1301, as the MHC class II restriction element for MSP1a peptide B. We also found that the product of the intrahaplotype-matched allelic pair, DQA* 2206/DQB*1402, was functional in presenting MSP1a peptide F3-5. This indicates that the *DQA*2206* allelic product plays a role in presenting at least two different CD4^{+} T-cell epitopes derived from MSP1a by pairing with *DQB* allelic products derived from the same and different haplotypes. In vitro expression of *DQA*2206/DQB*10021* in 293-F cells (Table 4) further suggests that the *DQA*2206* allele is flexible to pair with multiple *DQB* alleles and may play a dominant role for helper T-cell responses in animals carrying the DH22H haplotype. Further, we have identified functional DQ molecules, DQA*10011/DQB*10021, DQA* 12011/DQB*10051, and DQA*2201/DQB*1201, which present peptides MSP1a F3-3, MSP2 P25, and MSP2 P12-AM5, respectively. These functional DQ pairs are most likely intrahaplotype DQ molecules consisting of products of adjacent loci according to our linkage data. In this study, functional intrahaplotype DQ molecules consisting of products of nonadjacent loci were not identified, although this type of intrahaplotype DQ molecule was expressed on 293-F cells in some combinations. This is likely explained by the limited numbers of antigens tested in our study, and more extensive analysis may reveal that this type of intrahaplotype DQ molecule is also functional.

It is of interest that some DQA and DQB combinations were not detected on transfected 293-F cells. Preferential pairing of DQ chains has also been reported for humans and mice. With human cells, interchange between DQA and DQB chains occurs within the DQw1 family of haplotypes, but not between this family and the other non-DQw1-associated haplotypes, whereas DQw2-, 3-, and 4-associated haplotypes are freely interchangeable (Kwok et al. 1993). As in sheep, bovine class II *DQA*10011* and *DQA*12011* alleles are grouped in the *DQA1* cluster, while *DQA*22021*, *DQA*2206*, and *DQA*2201* alleles are

grouped in the *DQA2* cluster (Hickford et al. 2004; Chris Davies, personal communication). Our observation that products of *DQA*10011* and *DQA*12011* alleles paired with only one *DQB* allelic product is consistent with the possibility that *DQA* alleles within the *DQA1* cluster may have more limited structural constraints for pairing. However, additional studies are needed to confirm this possibility.

Additional evidence for functional interhaplotype pairing of DQA and DQB proteins is the observation that two CD4$^+$ T-cell clones specific for *B. bovis* Hsp20 peptide P1 were restricted by products of both *DQA*0301/DQB*0402* and *DQA*C97/DQB*0402* pairs. Because duplicated DQ alleles were not identified in this donor animal (C97), one of these allelic pairs appears to result from interhaplotype pairing. Unfortunately, the Brahman–Angus cross cow C97 expresses a rare BoLA haplotype, so it was not possible to prove this using haplotype homozygous APCs. However, this finding is similar to the report that some T-cell hybridomas specific for HEL in (H-2^k×H-2^b) F1 mice were also restricted by two different I-A molecules, I-Aα^{b}β^k and I-Aα^{k}β^k (Moreno et al. 1990). The ability of Hsp20-specific T-cell clones to respond to peptide presented by two different DQA/B heterodimers may be attributed to the high degree of homology between the two DQα chains, which are 94% identical in the amino acid sequence encoded by exon 2.

In humans, HLA-heterozygosity has drawn attention largely from association of HLA haplotype with immunologically mediated disease (McDevitt and Bodmer 1972; Todd et al. 1987; Nepom and Erlich 1991). For example, people with HLA-DQ2 (*DQA1*05/DQB1*02*) are strongly predisposed to celiac disease, and DQ2 can be generated by interhaplotype or intrahaplotype pairing (Sollid et al. 1989; Sollid 2002). Interestingly, a dose effect of HLA-DQ2 expression appears to confer susceptibility to developing celiac disease depending on the homozygous or heterozygous status of specific haplotypes containing *DQA1*05* and *DQB1*02* alleles (Vader et al. 2003). These reports indicate that MHC class II DQ molecules generated by interhaplotype A/B chain pairing may occur rather commonly in outbred species like humans and cattle, where most individuals express heterozygous haplotypes. Although association of diseases with BoLA haplotypes is largely unknown, several lines of evidence indicate that certain BoLA haplotypes are associated with resistance to persistent lymphocytosis caused by bovine leukemia virus infection (Lewin 1989; van Eijk et al. 1992b; Xu et al. 1993; Nagaoka et al. 1999). Furthermore, an association between mastitis and BoLA-class II haplotypes was observed (Park et al. 2004; Lundén et al. 1990; Starkenburg et al. 1997; Sharif et al. 1998), and two *BoLA-DRB3* alleles were significantly associated with protection against *Theileria parva* challenge (Ballingall et al. 2004). However, in spite of the clear indication that BoLA-DR and BoLA-DQ molecules may play a role in protective immunity or susceptibility to infectious disease, functional analysis of BoLA-class II has rarely been reported.

In summary, using a transient class II and CD80/CD86 expression system, this study provides evidence that BoLA-DQ molecules contribute to priming helper T cells against epitopes of important pathogens of cattle such as *B. bovis* and *A. marginale* by generating DQA/B heterodimers formed by interhaplotype and intrahaplotype pairing. These results and those of previous studies (Brown et al. 2001b, 2002, 2003, 2004; Norimine et al. 2002, 2004) indicate that CD4$^+$ T-cell responses against the proteins examined are not skewed toward DQ-restriction as observed in FMDV-immunized animals (Glass et al. 2000), but consist of both DR- and DQ-restricted responses. Nevertheless, it appears that some alleles play more dominant roles in antigen presentation than others. If protective immune responses against bovine pathogens are strongly influenced by certain MHC class II alleles, characterization of the role of BoLA-DQ is extremely important for effective vaccine development or breeding strategies.

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