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Characterization and allelic variation of the transporters associated with antigen processing (TAP) genes in the domestic dog (*Canis lupus familiaris*)

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Abstract

The function of the transporters associated with antigen processing (TAP) complex is to shuttle antigenic peptides from the cytosol to the endoplasmic reticulum to load MHC class I molecules for CD8+ T-cell immunosurveillance. Here we report the promoter and coding regions of the canine TAP1 and TAP2 genes, which encode the homologous subunits forming the TAP heterodimer. By sampling genetically divergent breeds, polymorphisms in both genes were identified, although there were few amino acid differences between alleles. Splice variants were also found. When aligned to TAP genes of other species, functional regions appeared conserved, and upon phylogenetic analysis, canine sequences segregated appropriately with their orthologs. Transfer of the canine TAP2 gene into a murine TAP2-defective cell line rescued surface MHC class I expression, confirming exporter function. This data should prove useful in investigating the association of specific TAP defects or alleles with immunity to intracellular pathogens and cancer in dogs.

Keywords

Alleles; Canine; Immunology; Splice variants; Transporter associated with antigen processing

1. Introduction

The development, homeostasis and function of CD8⁺ T cells depends on the interaction of the T cell receptor with MHC class I molecules presenting peptide epitopes derived from degraded intracellular proteins. An essential step in the antigen processing and presentation pathway is the shuttling of these cytosolic peptides across the ER membrane to ultimately access the extracellular compartment, a function performed with high efficiency by the transporters associated with antigen processing (TAP) complex. In a single mammalian cell

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Conflict of interest

The authors declare no conflict of interest.

under physiologic conditions, the TAP system can transport $>2 \times 10^4$ peptides per minute (Neefjes et al., 1993). While TAP-independent pathways for peptide loading have also been described (Henderson et al., 1992; Snyder et al., 1997), conventional TAP-mediated transport appears to be the dominant mechanism, as demonstrated by the almost complete loss of surface MHC class I expression in the absence of functional TAP (Gadola et al., 2000; Kelly et al., 1992; Powis et al., 1991; Van Kaer et al., 1992). An ATP-binding cassette (ABC) transporter super-family member, TAP is a heterodimeric complex composed of TAP1 and TAP2 subunits, which are structurally homologous. Each half-transporter has a transmembrane domain (TMD) and a cytosolic nucleotide-binding domain (NBD). Together, the six C-terminal transmembrane helices of each TMD form the TAP core complex necessary for peptide translocation, which is powered by the binding and hydrolysis of ATP at the NBDs (Procko and Gaudet, 2009).

The TAP loci lie within the class II region of the MHC (Debenham et al., 2005). In humans, the genes encoding each half-transporter are modestly polymorphic, with official recognition of six TAP1 and five TAP2 (protein level) alleles (<http://hla.alleles.org/classo.html>). Additional subtypes and splice variants (SVs) of each subunit have been described. Only a few amino acid changes scattered throughout the protein sequences distinguish alleles of TAP1 and TAP2, unlike the polymorphisms of classical MHC molecules, where far more numerous allelic differences are observed, concentrated in hypervariable regions. Importantly, some TAP alleles in humans have been correlated with increased susceptibility to immune-mediated (Barron et al., 1995; Gonzalez-Escribano et al., 1995; Ramos et al., 2009; Rau et al., 1997; Slomov et al., 2005) and infectious diseases (Rajalingam et al., 1997; Zhang et al., 2003), independent of linkage disequilibrium with class II genes. Whether these associations reflect altered CD8⁺ T-cell immunosurveillance is uncertain, as peptide selection and transport does not differ significantly between alleles (Daniel et al., 1997; Obst et al., 1995). Allelic variation of the TAP1 and TAP2 genes has also been described in the gorilla, rat, mouse, sea bass, and several avian species (Laud et al., 1996; Livingstone et al., 1991; Loflin et al., 1996; Marusina et al., 1997; Pinto et al., 2011; Sironi et al., 2008; Walker et al., 2011). The objectives of this study were to characterize the TAP genes of the domestic dog, and determine whether polymorphisms were present. Accordingly, we sequenced the coding regions of these genes obtained from dogs belonging to four genetically distinct breed clusters (Parker et al., 2004). Five TAP1 and four TAP2 alleles were found, discriminated by only a few amino acid substitutions. In several dogs, variants of both genes produced by alternative RNA splicing were also identified. The peptide exporter function of the canine TAP2 subunit was established by gene transfer into TAP2-defective murine RMA-S cells.

2. Materials and methods

2.1 Preparation of RNA and DNA samples

The canine histiocytic cell line DH82 (ATCC CRL-10389) was grown in Dulbecco's Modified Eagle Medium supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin (cellgro). For passage or harvest, cells were detached with 0.05% trypsin-EDTA. Venous blood anticoagulated with EDTA was obtained with owner consent from samples collected from unrelated adult dogs ($n = 10$) undergoing evaluation at the North Carolina State University Veterinary Teaching Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation ($400 g \times 30 \text{ min}$ at 22°C) over Histopaque 1.077 (Sigma-Aldrich). From PBMC or DH82 cell lysates, RNA was extracted using Qias shredder columns and the RNeasy Plus Mini kit (Qiagen). Complementary DNA was generated by reverse transcription (Omniscript RT kit, Qiagen) using an oligo(dT)₁₅ primer.

2.2 Amplification and cloning of TAP genes

For PCR, exonic TAP1- and TAP2-specific primers (Supplemental Table 1) were generated using canine genomic and expressed sequence tag data, and homologous TAP gene sequences available through the National Center for Biotechnology Information (NCBI) HomoloGene database (<http://www.ncbi.nlm.nih.gov/homologene>), and were synthesized by Invitrogen. The upstream primers were designed to anneal to the most highly conserved 5' regions of TAP1 and TAP2 across mammalian orthologs. Template cDNA was amplified with a HotStar HiFidelity Polymerase kit (Qiagen), using Q solution, on a Mastercycler Pro thermocycler (Eppendorf), programmed with the following cycling parameters: initial denaturation of 5 min at 95°C, followed by 35 cycles of 15 s at 94°C, 1 min at 65.1°C, and 3 min at 72°C, and a final elongation step for 10 min at 72°C. Amplification products were electrophoresed on a 1% agarose gel, and ~2.5kb bands for each TAP gene were excised and purified. Amplimers were TA cloned with pGEM-T Easy Vector (Promega), and colonies were screened by blue/white analysis and EcoRI digestion.

2.3 Sequencing and analyses of TAP1 and TAP2 alleles

Sanger DNA sequencing of insert-containing plasmids was performed by Eurofins MWG Operon, using standard primers (T7 and Sp6), as well as four internal primers for each gene, generating six total sequences per colony. Assembly of overlapping reads and alignment of concatenated sequences with the predicted TAP1-001 and TAP2 sequences-001 obtained from the canine BAC clone 58o15 (GenBank ID: AJ630364.1) were performed using Geneious v.5.1 software (Drummond et al., 2010). For each dog, a minimum of six colonies were sequenced per gene in order to demonstrate homozygosity with $>96\%$ $([1 - (0.5^{n-1})] \times 100)$ confidence. The amino acid sequences for canine TAP1 and TAP2 genes were deduced using Geneious. Alleles were defined as sequences containing non-synonymous nucleotide substitutions found in three or more colonies, typically corroborated by a second, independent PCR amplification. Alleles were named following the nomenclature convention for MHC genes in dogs, i.e., *001, *002, *003, with the allele number *001 arbitrarily assigned to previously predicted sequences (Debenham et al., 2005). Sequences validated by 3 colonies containing synonymous nucleotide changes were designated as subtypes, which was indicated by appending a letter to the allele number, e.g., *001B. A tree of TAP alleles was constructed on the basis of genetic distances (Tamura and Nei, 1993) using the neighbor-joining method (Saitou and Nei, 1987).

2.4 Determination of the 5' untranslated regions (UTRs) of TAP1 and TAP2 genes

RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE, FirstChoice®, Ambion) was performed on the 5' ends of TAP1 and TAP2 mRNA per the manufacturer's protocol. Briefly, RNA was isolated from DH82 cells that had been cultured for 16 hours with ~600 units/mL recombinant human interferon (IFN)- γ (Peprotech) to increase TAP gene transcript levels (Ma et al., 1997), and treated sequentially with calf intestinal alkaline phosphatase and tobacco acid pyrophosphatase, ligated to a 5' adaptor, and reverse transcribed. Amplification was performed by nested PCR, using 5' adaptor-specific primers and 3' gene-specific primers (Supplemental Table 1) and the HotStar HiFidelity Polymerase kit. Cycling conditions were as follows: initial denaturation of 5 min at 95°C, followed by 35 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final elongation step for 7 min at 72°C. After TA cloning of PCR products, plasmids were sequenced for each TAP gene.

2.5 Cloning and expression of the canine TAP2 gene in a TAP-defective murine cell line

The canine TAP2*001 allele was amplified from cDNA of IFN- γ -treated DH82 cells using the thermocycler conditions described in section 2.2; primers are listed in Supplementary

Table 1. The gel-purified PCR product was ligated into a pEgfp-C2 expression vector at the BamHI and NotI restriction sites to create an N-terminal green fluorescent protein (GFP) fusion protein. The murine lymphoma RMA-S cell line, which was grown in RPMI-1640 medium supplemented to 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin, was transfected with the pEgfp-TAP2 and parental pEgfp-C2 constructs using the Amaxa Nucleofector Kit T solution and the A-030 program. Twenty-four hours later, cells were placed in medium containing G418 (1 mg/ml; Invitrogen). Following 2 weeks of selection, surface expression of MHC class I molecules was determined by staining cells cultured at 37°C with anti-H2-D^b (28-12-8, BioLegend) and anti-H2-K^{b/d} (34-1-2S, eBioscience) antibodies (Abs). An Alexa Fluor 647-labeled donkey anti-mouse IgG Ab (Jackson ImmunoResearch) was used for detection; background fluorescence was established by omitting the primary Ab. Cell viability was discriminated on the basis of forward and side scatter. Flow cytometric list mode data were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with Summit software v5.2 (Beckman Coulter).

3. Results and Discussion

3.1 Polymorphisms of TAP1 and TAP2 sequences in the dog

Using PBMCs from a mixed-breed dog, we first determined the coding sequences of each of the TAP subunit genes, which have not been previously reported. Alignment of these sequences showed >99% identity with the predicted TAP1 and TAP 2 sequences generated from a genomic BAC clone of the Dog Leukocyte Antigen (DLA) class II region (GenBank ID: AJ630364.1) (Debenham et al., 2005). For TAP1, expressed sequence tag (EST) representation of all 11 exons was found (20 ESTs with 97% identity); for TAP2, 11 ESTs with 97% identity to our sequences covered all exons, except for exons 5-8 (bp 877 to 1289).

While polymorphisms have been described in the TAP genes of other species, the number of alleles is generally few, and consequently, it was uncertain whether any variants would be found in dogs unless a large number of individuals were sampled. Moreover, diversity at canine MHC loci is relatively restricted, with low levels of heterozygosity and allelic variation, as a result of population bottlenecks that occurred with domestication. For example, at the DLA-DRB1 locus, only 100 alleles have been described (Kennedy et al., 2007), in contrast to the >1050 known Human Leukocyte Antigen (HLA)-DRB1 alleles (<http://www.ebi.ac.uk/imgt/hla/stats.html>). Accordingly, to increase the likelihood of TAP allele discovery, we obtained samples from two or more representative dogs from four divergent breed clusters: Ancient-Asian (Akita; Chow Chow; Shar-Pei), Herding-Sighthound (Greyhound; Shetland Sheepdog), Mastiff-like (Mastiff; Rottweiler), and Hunting (Airedale Terrier; Doberman Pinscher) (Parker et al., 2004). In humans, TAP allele usage has been shown to vary between ethnic populations (Faucz et al., 2000). Non-synonymous single nucleotide polymorphisms (SNPs) were found in the sequences of both canine TAP genes. Alignments of the amino acid sequences of these alleles are shown in Fig. 1A (TAP1) and 1B (TAP2). As in humans, the number of amino acid changes between variants is very low and distributed across exons. In addition to these alleles, SNPs that resulted in synonymous mutations were also found in our dogs, which were designated as allele subtypes (Table 1).

A list of alleles and the corresponding dog breed from which they were obtained is provided in Table 2. Consistent with limited diversity, only two heterozygotes were noted; interestingly, both individuals were in the Ancient-Asian group. Members of this cluster (Akita; Chow Chow; Basenji) appear to have lower homozygosity across several MHC class II loci than other breeds (Angles et al., 2005), and are considered representatives of the ancestral gene pool of dogs (Parker et al., 2004). The *001 allele was the most prevalent

variant of each gene: six of eight dogs carried TAP1*001/*001B and five of eight dogs carried TAP2*001/*001B. The TAP *001 sequences were originally identified from the RCPI-81 canine BAC library, prepared from a Doberman Pinscher. Hence, we included the same breed in this analysis; interestingly, our dog varied at both loci, bearing the TAP1*006 and TAP2*004 alleles.

3.2 Splice variants of canine TAP1 and TAP2 genes

Variants of both canine TAP genes resulting from alternative splicing were observed occasionally. Splice variants of TAP genes have been found in humans, pigs and sea bass (Furukawa et al., 1999; Garcia-Borges et al., 2006; Pinto et al., 2011; Yan et al., 1999). The most common variant, TAP2*001 SV1, was identified in two dogs (five colonies) and is depicted in Fig. 2A. The large deleted region in TAP2*001 SV1 appears to be a genuine product of alternative splicing, rather than an artifact caused by template switching during reverse transcription, as the exonic donor and acceptor sequences within the SV match 5' and 3' splice site consensus sequences (Mount, 1982). Several other TAP1 and TAP2 SVs were also found that appeared to be generated by exonic skipping or retention of intronic sequences, but were represented by only 1 or 2 colonies, and were not pursued further.

In humans, TAP gene SVs can have demonstrable functional consequences. The peptide selectivity of a TAP2 SV was shown to be qualitatively different than that of the standard allele (Yan et al., 1999). In a more dramatic example, a point mutation in the acceptor site at the 3' end of intron 1 of TAP1 generated an SV with a frameshift and premature stop, resulting in the virtual lack of cell surface MHC class I expression, a condition known as Bare Lymphocyte Syndrome (Furukawa et al., 1999). The two TAP1 SVs that we found had small changes that lay outside of conserved functional regions and did not alter the reading frame. On the other hand, the TAP2*001 SV1 had deletions in sites important for peptide binding (residues 414-433) and interaction with ATP (residues 503-510), as well as a premature stop (Fig. 2A). Nonetheless, TAP2*001 SV1 presumably held no deleterious consequences for the dogs carrying this variant, as the wild-type allele was also amplified from these individuals.

3.3 Comparative analysis of 5' UTR and flanking regions of canine TAP genes

We next wished to compare canine TAP genes with their counterparts from other species. To verify the predicted sequence of the 5' UTR, we performed 5' RACE, using RNA isolated from a canine histiocytic cell line, which expresses normal amounts of MHC class I molecules on the cell surface (not shown) and is heterozygous at both TAP loci (TAP1*001, *006; TAP2*001, *004 [DH82 cells are derived from a Golden Retriever (Wellman et al., 1988), a breed in the Hunting cluster]). The inner amplification product appeared as a single band (Fig. 2B), which was TA cloned. From a minimum of six colonies from each PCR, identical sequences of 631 bp (TAP1) and 516 bp (TAP2) were obtained, indicating a single transcription start site (TSS) for both, which differed from predicted sites. Typical of genes whose promoters lack a TATA box, transcription of TAP1 and TAP2 is usually initiated from multiple sites (Arons et al., 2001; Kishi et al., 1993; Wright et al., 1995). The finding of a single TSS may represent an idiosyncrasy of the DH82 cells, the effect of IFN- γ , or both; for example, Arons et al. (2001) found multiple TSSs in murine TAP2 using a T-cell leukemia line, but similarly, observed only one TSS in transcripts from IFN- γ -treated transformed fibroblasts. Multiple start site element downstream (MED)1 sequences (GCTCCC/G) were found in both TAP1 and TAP 2, which are common elements in TATA-less promoters that have multiple initiation sites, so presumably other TSSs are used in canine TAP genes, but 5'-RACE analysis of other canine cell lines will be needed to confirm this supposition.

For TAP1, 5' RACE data from our transcripts showed a UTR of 202 bp, with the first exon 147 bp shorter than predicted in the annotated genomic sequence, as depicted in Fig. 2B (lower left panel). The TAP2 gene has a 5' UTR of 39 bp. The 3' end of exon 1 is 86 bp shorter than predicted (Supplemental Fig. 2B, lower right panel), and similar to TAP2 of other species (Arons et al., 2001), there is a short 5' stretch of 8 nucleotides (7-8 in rodents; 5 in humans) in exon 2 before the translation initiation codon.

We then examined the 5' UTR (RACE data) and upstream flanking regions (GenBank genomic data) for promoter elements (in addition to MED1) that have been identified previously in murine and human TAP genes, using the search function in Geneious, and allowing for interpretations of ambiguities within query and sequence. Most notable among these *cis* elements are those related to responsiveness to IFN- γ . This inflammatory cytokine is an important bridge between innate and adaptive immune responses, possessing the well-established effects of increasing the expression of surface MHC class I molecules and several components of antigen processing pathway, including the TAP subunits, to enhance cytotoxic CD8+ T-cell activity. Interferon- γ signaling can modulate gene expression by generating phosphorylated Signal Transducer and Activator of Transcription (STAT)1 homodimers that bind the Gamma Activating Sequence (GAS) (Saha et al., 2010). Such responses are subsequently amplified by GAS-regulated transcription of Interferon Regulatory Factors (IRFs), which can bind the Interferon-stimulated response elements (ISRE) to promote expression of additional immune genes. Additionally, IFN- γ can regulate gene expression by indirectly activating NF- κ B through protein kinase R (PKR) (Deb et al., 2001). All three of the target elements potentially responsive to IFN- γ – GAS, ISRE and the NF- κ B element – were identified in both canine TAP sequences; a comparison to mouse and human TAP promoters is shown in Table 3.

In TAP1, a presumptive GC box (GCCCCGCCCCGCT) was found 315 bp upstream of the TSS; in human TAP1, there are two such elements (GCCCCGCCCCCT) that bind the transcription factor Specificity Protein (SP)1 and are important for regulating basal expression of the gene (Wright et al., 1995). On the other hand, the cAMP response element (TGAC/AGTCA) common to the 5' UTR of human TAP2 and both murine TAP genes (Arons et al., 2001) was not identified in our canine sequences, nor did we find the CCAAT/enhancer binding protein transcription factor element (GATTTGCGCAATCTGC; consensus: A/GTTGCGC/TAAC/T) that had been observed in the TAP2 promoter sequence of rainbow trout (Castro et al., 2008).

3.4 Comparative analysis of structural and functional elements and polymorphisms of canine TAP genes

The genomic organization of the TAP genes of the dog is essentially identical to that of the mouse and human (Marusina et al., 1997). The canine TAP1 gene has an open reading frame (ORF) of 2253 bp, with 11 exons. The canine TAP2 gene has an ORF of 2112 bp, with 12 exons; the first is non-coding. The predicted amino acid sequences of canine TAP1*001 and TAP2*001 were aligned with the orthologous genes from four other mammalian species. As expected, the major functional TAP elements were identified, as shown in the annotations of Fig. 3, with varying degrees of homology across domains. For example, when compared to murine TAP1, the canine protein shares 62.8% overall identity in the TMD, 74.6% in the NBD, 70.4% in the peptide-binding regions, and 100% identity in the C- and D-Loops and Coupling Helix 2. A comparable analysis for TAP2 reveals 100% identity in the Coupling Helices, Walker A and B motifs, and the C- and D-Loops, but differences in the peptide-binding regions (62.5% identity), X-Loop and H-Switch.

While the major peptide-binding regions of both TAP subunits reside both in the Cytosolic Loop (CL)2 domains and an stretch of 15 amino acids immediately following the last C-

terminal transmembrane helix of the TMD (Nijenhuis and Hammerling, 1996), four residues recently have been identified in the CL1 of human TAP1 that are involved in the sensing of bound peptides (V288) and inter-domain transmission of this signal (G282; I284; R287) (Herget et al., 2007). These residues are conserved in all alleles of the canine ortholog (Fig. 3). While an analogous sensor has not been reported for human TAP2, a cysteine at position 213 (beginning of CL2) has been shown to be important for orientation of peptides in the binding pocket, thereby influencing peptide selectivity (Baldauf et al., 2010). The residue C213 is also present in the dog, but interestingly, neither in rodents nor cattle (Fig. 3). Other TAP2 residues that have been shown to alter transporter specificity by controlling the rate of peptide export, based primarily on side-chain properties (hydrophobicity and polarity/charge) of the C-terminal amino acid, have been mapped to the CLs at positions 217/218 and 374/380 (Armandola et al., 1996; Momburg et al., 1996). As seen in Fig. 3, dogs share M218 and A374 with humans, and have an R380Q substitution, identical to rat 2a. The rat alleles 2a (also designated 2^a) and 2b (2^b) have different peptide specificities (Momburg et al., 1994). Similar to the 2b variant, mouse TAP has a strong preference for hydrophobic residues and an aversion to positively charged amino acids at the C-terminus. In contrast, the human TAP transporter resembles the less stringent selectivity of the rat 2a allele, accepting C-terminal hydrophobic and basic residues (Momburg et al., 1996). Based on these key TAP2 residues alone, canine TAP might be expected to show affinities similar to human TAP. However, a more recent study has emphasized the important added contribution of the three N-terminal amino acids of the peptide substrate towards human TAP specificity (Burgevin et al., 2008). As the particular TAP sites that interact with these residues have not been identified, a comparison between human and dog sequences is precluded, and consequently, it is difficult to predict the selectivity of the canine transporter. Moreover, dogs uniquely have an isoleucine at position 217 (most orthologs use a threonine). Finally, the TAP1 subunit also contributes to peptide selectivity (Armandola et al., 1996), so the exporter preferences of canine TAP ultimately will require empirical determination.

Four polymorphisms, S125C, L180P, A353S and P749T/L, differentiate the five canine TAP1 alleles (Fig. 1; Fig. 3), but all these positions are invariant across the six known human alleles (http://hla.alleles.org/data/txt/tap1_prot.txt). Conversely, none of the human TAP1 polymorphic sites (V80G, L131P, I333V, A370V, V458L, V518I, D637G and R648Q) were observed to vary in the deduced sequences from our dogs. All of the canine TAP2 sequences encoded the C-terminal stretch of amino acids 688 – 703 present in human *02 alleles but absent in *01 group members. However, there was no overlap between canine (G127R, R373C, I425T and R695H) and human (V379I, A565T, R651C and Q665A) polymorphisms (http://hla.alleles.org/data/txt/tap2_prot.txt). Similarly, examination of the five TAP1 and six TAP2 alleles in mice (Marusina et al., 1997) revealed that only one TAP2 polymorphic site (N425S) was shared with the dog (I425T).

A phylogenetic analysis of TAP sequences shows the expected clustering of the canine alleles with their orthologs from rodents, primates and the cow (Fig. 4).

3.5 Functional assessment of the canine TAP2 gene

With loss of function of either TAP subunit, the export of cytosolic peptides into the ER lumen for loading onto class I heavy chain-β2M light chain dimers is halted. Occupied instead by low-affinity self-peptides that readily dissociate from the binding groove (De Silva et al., 1999), or simply empty, the MHC class I molecules transported to the cell surface are structurally unstable and rapidly disappear (Kelly et al., 1992; Van Kaer et al., 1992). This phenomenon allows the function of either TAP molecule to be conveniently demonstrated by rescue of surface class I expression – as measured by flow cytometry – upon gene transfer into cells that have a defect in the corresponding subunit. Murine RMA-S cells, generated by chemical mutagenesis and negative selection (Ljunggren and Karre,

1985), have intact TAP1 but a truncated, defective TAP2 molecule (Yang et al., 1992), and are sometimes employed for this purpose. We therefore sought to use this system to test the function of canine TAP2. Because of the strong conservation of TAP functional elements across species (Fig. 3), hybrid partnering of orthologous subunits can generate a working heterodimer, as has been shown for various combinations of human, mouse and rat TAP1 and TAP2 molecules (Armandola et al., 1996; Powis et al., 1991; Yewdell et al., 1993). In analogous fashion, we cloned the TAP2 gene (*001 allele) into a GFP expression vector for transfection into RMA-S cells. As seen in Fig. 5, GFP+ cells containing the empty vector have low expression of H2-D^b (A) and -K^b (B), identical to unmanipulated RMA-S cells (not shown), while GFP+ cells complemented with canine TAP2 have markedly increased surface expression of both H-2 class I molecules, consistent with the restoration of peptide export into the ER. The canine TAP2 gene therefore encodes a functional transporter subunit capable of pairing with murine TAP1 to produce a competent TAP heterodimer.

4. Conclusions

Here we provide the first description of the promoter regions and coding sequences for the canine TAP1 and TAP2 genes. All highly conserved functional elements common to ABC exporters were identified. As in other species, alleles and subtypes of both genes are observed. Whether these variants have functional significance will require additional investigation. In humans, the association of TAP alleles with some autoimmune and infectious diseases suggests that polymorphisms are functionally important, while studies of peptide selectivity (Daniel et al., 1997; Obst et al., 1995) and genotype distribution (Faucz et al., 2000) do not. In the dog, the very limited amino acid variations of TAP1 and TAP2 alleles imply that effects on immune function and disease susceptibility, if any, will be modest at best. TAP variants generated by alternative splicing, on the other hand, can have more profound consequences, such as altered peptide selectivity and enhanced susceptibility to bacterial and viral infections, as seen in humans (Furukawa et al., 1999; Yan et al., 1999). A canine TAP2 SV that contained a stop codon early in the NBD coding sequence was discovered; however, the coexistence of its full-length allelic counterpart (*001), which was capable of replacing a defective murine ortholog in RMA-S cells, suggested that peptide export and class I loading would be largely unimpaired by the presence of this variant transcript. Finally, the most significant clinical relevance of the TAP transporter is perhaps the acquired dysfunction of the TAP1 or TAP2 subunits that is observed in some human persistent viral infections and cancers, as a mechanism for avoiding detection by CD8+ T cells (Ritz and Seliger, 2001). Investigating whether analogous processes of immunoevasion occur in canine malignant and virally infected cells should be assisted by the data from this study and will be important in fully understanding such disease processes in the dog.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in an online version, at the following URL: To be supplied.

Abbreviations

ABC	ATP-binding cassette
Ab	antibody
β2M	β2-microglobulin
CL	Cytosolic Loop
CDS	code determining sequence
DLA	Dog Leukocyte Antigen
EST	expressed sequence tag
IFN	interferon
IFR	Interferon Regulatory Factor
ISRE	IFN-stimulated response element
GAS	Gamma Activating Sequence
GFP	green fluorescent protein
HLA	Human Leukocyte Antigen
MED1	multiple start site element downstream1
NBD	nucleotide-binding domain
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PKR	protein kinase R
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
SNP	single nucleotide polymorphism
STAT	Signal Transducers and Activators of Transcription
SV	splice variant
TAP	transporter associated with antigen processing
TMD	transmembrane domain
TSS	transcription start site
UTR	untranslated region

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Highlights

Genes for peptide exporter subunits TAP1 & TAP2 were sequenced from cDNA of dogs.

Canine TAP genes display limited polymorphisms across divergent breed clusters.

Promoter & functional elements common to TAP genes are found in canine sequences.

Canine TAP2 can partner with murine TAP1 to restore MHCI expression on RMA-S cells.

A TAP1

Predicted *001 (AJ630364)	1	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
*003 (JN656393)	M	A	G	S	G	P	S	G	G	R	F	C	A	P	R	A
*004 (JN656394)	M	A	G	S	G	P	S	G	G	R	F	C	A	P	R	A
*005 (JN656396)	M	A	G	S	G	P	S	G	G	R	F	C	A	P	R	A
*006 (JN656397)	M	A	G	S	G	P	S	G	G	R	F	C	A	P	R	A
Predicted *001 (AJ630364)	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	
*003 (JN656393)	A	A	L	W	H	K	L	S	G	I	M	A	P	P	T	
*004 (JN656394)	A	A	L	W	H	K	L	S	G	I	M	A	P	P	T	
*005 (JN656396)	A	A	L	W	H	K	L	S	G	I	M	A	P	P	T	
*006 (JN656397)	A	A	L	W	H	K	L	S	G	I	M	A	P	P	T	
Predicted *001 (AJ630364)	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	
*003 (JN656393)	E	K	L	S	L	L	M	V	P	R	G	L	L	G	I	
*004 (JN656394)	E	K	L	S	L	L	M	V	P	R	G	L	L	G	I	
*005 (JN656396)	E	K	L	S	L	L	M	V	P	R	G	L	L	G	I	
*006 (JN656397)	E	K	L	S	L	L	M	V	P	R	G	L	L	G	I	
Predicted *001 (AJ630364)	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600	
*003 (JN656393)	Q	I	Q	T	S	A	V	E	A	L	F	S	T	P	S	
*004 (JN656394)	Q	I	Q	T	S	A	V	E	A	L	F	S	T	P	S	
*005 (JN656396)	Q	I	Q	T	S	A	V	E	A	L	F	S	T	P	S	
*006 (JN656397)	Q	I	Q	T	S	A	V	E	A	L	F	S	T	P	S	
Predicted *001 (AJ630364)	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	
*003 (JN656393)	Y	G	L	V	K	P	T	M	E	E	V	I	A	A	M	
*004 (JN656394)	Y	G	L	V	K	P	T	M	E	E	V	I	A	A	M	
*005 (JN656396)	Y	G	L	V	K	P	T	M	E	E	V	I	A	A	M	
*006 (JN656397)	Y	G	L	V	K	P	T	M	E	E	V	I	A	A	M	

B TAP2

	1	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150
Predicted *001 (AJ630364)	M	R	P	D	L	R	F	M	A	P	L	L	A	D	W	L
*002 (JN656402)	M	R	P	D	L	R	F	M	A	P	L	L	A	D	W	L
*003 (JN656403)	M	R	P	D	L	R	F	M	A	P	L	L	A	D	W	L
*004 (JN656400)	M	R	P	D	L	R	F	M	A	P	L	L	A	D	W	L
	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	
Predicted *001 (AJ630364)	Y	L	V	A	A	F	F	L	V	A	V	L	G	E	T	V
*002 (JN656402)	Y	L	V	A	A	F	F	L	V	A	V	L	G	E	T	V
*003 (JN656403)	Y	L	V	A	A	F	F	L	V	A	V	L	G	E	T	V
*004 (JN656400)	Y	L	V	A	A	F	F	L	V	A	V	L	G	E	T	V
	310	320	330	340	350	360	370	380	390	400	410	420	430	440	450	
Predicted *001 (AJ630364)	P	L	A	I	T	A	K	V	Y	M	V	H	E	A	V	L
*002 (JN656402)	P	L	A	I	T	A	K	V	Y	M	V	H	E	A	V	L
*003 (JN656403)	P	L	A	I	T	A	K	V	Y	M	V	H	E	A	V	L
*004 (JN656400)	P	L	A	I	T	A	K	V	Y	M	V	H	E	A	V	L
	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600	
Predicted *001 (AJ630364)	P	N	L	H	P	P	G	T	L	A	P	P	T	L	G	L
*002 (JN656402)	P	N	L	H	P	P	G	T	L	A	P	P	T	L	G	L
*003 (JN656403)	P	N	L	H	P	P	G	T	L	A	P	P	T	L	G	L
*004 (JN656400)	P	N	L	H	P	P	G	T	L	A	P	P	T	L	G	L
	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	
Predicted *001 (AJ630364)	G	E	K	G	N	L	A	V	G	K	Q	C	L	A	I	A
*002 (JN656402)	G	E	K	G	N	L	A	V	G	K	Q	C	L	A	I	A
*003 (JN656403)	G	E	K	G	N	L	A	V	G	K	Q	C	L	A	I	A
*004 (JN656400)	G	E	K	G	N	L	A	V	G	K	Q	C	L	A	I	A

Fig. 1.

Alignment of the amino acid translations of the TAP1 and TAP2 sequences recovered from dogs in this study with predicted GenBank sequences reveals limited polymorphisms in canine alleles. (A) Translated TAP1 sequences show four residue changes (letters; dots signify identities). The absent TAP1*002 designation was reserved for a TAP1-002 splice variant predicted by the data of Debenham et al. (2005) and was not found in this study. (B) Translated TAP2 sequences also display substitutions at four positions. The *001 alleles that we obtained for both genes match their reference sequences and are not shown. GenBank accession numbers are indicated in parentheses.

Fig. 2.
 (A) Schematic representation of the code determining sequence (CDS) for the TAP2*001 SV1 (GenBank ID: JN656405). Exons are depicted by boxes; the gray areas constitute the 5' untranslated region. The SV1 transcript contains a 53 bp deletion at the 3' end of exon 7 through exon 8 (red). Transcription is resumed at the beginning of exon 9, resulting in a frame shift and creation of a premature stop codon (symbol). The functional domains affected by splicing are shown above in the full-length TAP2*001 (GenBank ID: AJ630364.1). Introns (connecting lines) are placed for clarity and are not drawn to scale. (B) The 5' UTR of canine TAP1 and TAP2 as determined by RLM-RACE. Electrophoresis of nested PCR products revealed a single band per reaction, which was TA cloned and

sequenced; all colonies from each gene returned the same nucleotide sequence (GenBank ID: JN656391 [TAP1]; JN656400 [TAP2]). MW – molecular weight ladder. Lower panels: Schematic alignment of the 5' RACE products (shown in red) with the corresponding predicted TAP1 and TAP2 sequences (GenBank ID: AJ630364.1; shown in black). The translation start sites are indicated by the black arrows and ATG codon.

A TAP1

B TAP2

Fig. 3.

Alignment of the deduced amino acid sequences of the TAP genes of the dog with orthologs from four mammalian species reveals strong conservation of all major functional elements. The most common canine allele at each locus (*001) was used for comparison; sites of polymorphisms are indicated by red arrows. For optimal alignment, the proline at position 23 of the rat TAP1 protein was omitted (red symbol); other gaps are shown as dashes. Dots signify identities; letters indicate substitutions. Annotations of functional regions, shown in the black bars below the sequences, are based on descriptions for human TAP proteins (Parcej and Tampe, 2010). Excluding the transmembrane domain, the greatest percentage differences between the canine and human sequences were observed in the H-Loop of TAP1, and in the peptide-binding regions of TAP2. (A) Canine TAP1 exhibits 72.8, 72.8, 72.8, and 74.7% pairwise identity with the human, rat, mouse and cow genes, respectively. (B) Canine TAP2 exhibits 78.7, 72, 73.5, and 78.9% pairwise identity with the human, rat, mouse and cow genes, respectively. The accession numbers for these sequences are listed in Supplemental Table 2.

Fig. 4. Phylogenetic relationships of TAP1, TAP2 and TAPL (ABCB9) transporter proteins from various mammalian species. Predicted and actual protein sequences from public databases (see Supplemental Table 2 for accession numbers) were aligned and trimmed to the deduced TAP1 and TAP2 amino acid sequences from dogs in this study. An unrooted tree was built with Geneious 5.1, based on genetic distances, using the neighbor-joining method. Branch numbers are confidence values (%) based on bootstrap sampling (1000 replicates). Scale bar: number of nucleotide substitutions per site.

Fig. 5.

Expression of surface MHC class I is restored in murine RMA-S cells by the canine Tap2 gene. Gray histograms show cells transfected with the empty GFP vector, and those in black represent cells that received the GFP vector encoding TAP2. Flow cytometric overlays are gated on live GFP⁺ cells. Filled histograms indicate staining with 2° Ab only. A) H2-D^b; B) H2-K^b. Histograms represent four independent analyses.

Table 1

Sites of synonymous SNPs in allele subtypes.

Gene	Subtype	CDS		3' UTR		GenBank ID
TAP1	*001B	C597T ^a	T765C	C2271T	G2306A	JN656392
	*004B	G129C	C597T			JN656395
TAP2	*001B	G243C				JN656401
	*004B	T1566C	T1719C	A2189T	C2211T	JN656404

^a Nucleotide substitutions are numbered relative to the start codon.

Table 2

Alleles carried by dogs in this study.

Gene	Cluster ^a	Breed	Alleles	
TAP1	Ancient Asian	Akita Shar-Pei	*003 *001	*004 *005
	Herding-Sighthound	Greyhound Shetland Sheepdog	*001B *001	
	Hunting	Airedale Terrier Doberman Pinscher	*001 *006	
	Mastiff-like	Mastiff Rottweiler	*001B *001	
TAP2	Ancient Asian	Akita Chow Chow	*002 *004B	*003
	Herding-Sighthound	Greyhound Shetland Sheepdog	*001 *001	
	Hunting	Airedale Terrier Doberman Pinscher	*001 *004	
	Mastiff-like	Mastiff Rottweiler	*001B *001	

^aGenetic clusters were defined by Parker et al. (2004).

Table 3*Cis* elements in TAP promoters that are potentially responsive to IFN- γ

Gene	Species	GAS ^a	ISRE ^b	NFkB-E	References
TAP1	Mouse	+/- ^c	+	+/-	Arons et al. 2001; Kishi et al. 1993; Saha et al. 2010; White et al. 1996
	Human	+	+	+	Min et al. 1996; Wright et al. 1995
	Dog	+ ^d	+ ^e	+ ^{d,e}	This study
TAP2	Mouse	+	+	-	Arons et al. 2001; Guo et al. 2002; Saha et al. 2010
	Human	+	-	+	Saha et al. 2010
	Dog	+	+	+	This study

^a Gamma Activating Sequence (GAS): TTNCNNNAA; IFN-stimulated response element; (ISRE): AGTTTCNNTTTC/TCC or NGAAANNGAAAG/CN; NFkB-Element (NFkB-E): GGGA/GNNC/TC/TCC (sequences from Saha et al. 2010).

^b Highly homologous to sequences reported as: IFN Regulatory Factor (IRF)1/IRF2 binding element (Arons et. al); IRF-enhancer (IRF-E) (White et al.); IFN response factor binding element (IRFE) (Guo et al.); and IFN Consensus Sequence (ICS) (Arons et. al; Min et al.).

^c The +/- symbol is shown when in silico analyses disagree and no empirical data is available.

^d Observed in our 5'-RACE sequences; the remainder of canine elements listed in the Table were identified in the genomic sequence (GenBank ID: AJ630364.1) 5' to the TSS.

^e Two elements were found.