ORIGINAL ARTICLE

Mouse strains with point mutations in TAP1 and TAP2

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We report two new mouse strains: Jasmine (C57BL/6J/Abp-Tap2jas/Abp), with a point mutation in the transporter associated with antigen processing (TAP)2; and Rose, (C57BL/6J/Abp-Tap1rose/Abp), with a point mutation in TAP1. These strains were detected as the result of ethyl nitroso urea (ENU) screens for recessive point mutations affecting the immune system. As expected in cases of defective TAP expression, the mice have very low major histocompatibility complex (MHC)-I cell-surface expression, and few CD8⁺ T cells. The Rose strain has an A to T substitution in exon 10 of TAP1, resulting in an asparagine to valine substitution at position 643. Jasmine has an A to C transversion in exon 5 of TAP2, resulting in a threonine to proline substitution at position 293 of the protein. The mutation does not affect mRNA levels, but results in a very severe reduction in TAP2 protein. TAP1 protein levels are also decreased in Jasmine mice, demonstrating a new role for mouse TAP2 in stabilizing TAP1 protein expression. Jasmine is the first strain available with defective TAP2. The two mouse strains provide additional animal models for the human condition Bare Lymphocyte syndrome type 1, and identify new residues important for TAP function.

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The transporter associated with antigen processing (TAP) has a key role in antigen presentation to cytotoxic T cells. TAP is made up of TAP1 and TAP2 transmembrane proteins. The TAP heterodimer is located in the membranes of the endoplasmic reticulum (ER) and cis-golgi.¹ TAP transports peptides produced in the cytosol by the 20S proteasome to the ER lumen. Here, the peptides can bind major histocompatibility complex class I (MHC)-I proteins for transport to the cell surface and presentation to CD8⁺ T cells.^{2,3} The MHC-I heterodimer, a non-covalent interaction between a polymorphic MHC-I α chain and a non-polymorphic β 2 microglobulin chain, is stable only when bound by peptides. Thus, by supplying MHC molecules with peptides, TAP allows for the formation of a stable peptide-MHC-I complex.⁴

Both TAP proteins belong to the ATP-binding cassette family of transporters, which all share the same basic architecture. A functional transporter (for example, a TAP1/TAP2 heterodimer) consists of two nucleotide binding domains and two transmembrane domains.⁵ Comparisons with other ATP-binding cassette transporters and hydrophobicity analyses reveal that the TAP1 and TAP2 molecules consist of 10 and 9 transmembrane segments, respectively.^{6,7} The transmembrane domains are linked to the cytoplasmic nucleotide binding domains that contain conserved ATP-binding, or Walker A and B, motifs.⁸

Adenosine triphosphate hydrolysis is an absolute requirement for peptide translocation^{9,10} and there is an evidence suggesting that ATP hydrolysis mediated by TAP2, but not TAP1, is essential for both

peptide binding and translocation.^{11,12} There is an optimal peptide length for TAP-mediated translocation (8–16 amino acids),¹³ with differences in the affinity that TAP has for certain peptides influencing the types of peptides presented by MHC-I.¹⁴ TAP2 influences the transport specificity of peptides with different C-terminal amino acids, and point mutations in TAP2 can markedly alter the spectrum of peptides transported.^{15,16}

The importance of the TAP proteins for antigen presentation to CD8⁺ T cells has been demonstrated both in cell lines, and in knockout mice. Restoration of TAP1 and TAP2 in cell lines deficient in these molecules completely restored the ability of these cells to present antigen to T lymphocytes.^{17,18} Two independent lines of TAP1 knockout mice have been generated.^{19,20} Both show very low levels of cell surface MHC-I expression, and a very substantial reduction in CD8⁺ T-cell numbers. This phenotype is similar to that of β 2-microglobulin knockout mice, which are also deficient in MHC-I expression and circulating CD8⁺ T cells,^{21–24} demonstrating the critical role TAP has in the MHC-I antigen processing and presentation pathway.

Underscoring the importance of TAP proteins in cytotoxic T-cell responses, several herpes viruses, as well as adenoviruses and a human papilloma virus are known to modulate TAP levels or function during infection.^{25,26} Several of these viruses produce proteins which specifically interfere with TAP function, others interfere with transcript levels or phosphorylation levels.^{25,27} Furthermore, decreased TAP expression is often found in cancerous cells.^{25,26,28}

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Mutations in TAP proteins have been found in human patients, resulting in the disease Bare Lymphocyte syndrome type I,²⁹ characterized by the reduction in surface MHC-I expression. In this clinically heterogeneous disease, patients surprisingly do not usually suffer from severe viral infections, but most often suffer from recurrent bacterial infections of the respiratory tract starting at 4–7 years of age, and in around a third of cases, from granulomatous skin lesions.²⁹

In this study we describe the characterization of two new strains of mice, both lacking TAP function, 'Jasmine' has a point mutation in TAP2, and the recently identified 'Rose' has a point mutation in TAP1.

RESULTS

Phenotype of the Jasmine mice

The Jasmine mouse strain was identified as part of an ethyl nitroso urea screen for mice with recessive immunological phenotypes.^{30,31} The mice were derived on the C57Bl/6 background (H-2^b). The strain was originally identified because of a reduced percentage of CD8⁺ T cells in peripheral blood. The phenotype was found to be heritable and fully penetrant. Reduced percentages of CD8⁺ lymphocytes were also seen in the spleen of Jasmine mice, and reduced percentages of single-positive CD8 cells were seen in the thymus (Figure 1). As shown in Figure 1, the reduction in CD8⁺ cells was comparable to that seen in β_2 -microglobulin knockout mice, although Jasmine mice had slightly higher percentages of developing CD8 SP thymocytes than β_2 -microglobulin knockout mice. Cell-surface MHC-I expression was quantified by flow cytometry using antibodies against H-2D^b and H-2K^b (Figure 2). Jasmine mice were found to express substantially lower levels of MHC-I than wild-type mice, with levels comparable to those seen in β_2 -microglobulin knockout mice.

Mapping and identification of the Jasmine mutation

The causative mutation was mapped to the MHC region on chromosome 17 by crossing the Jasmine mice to the NOD^k mapping

strain. The progeny of Jasmine $\times$ NOD^k (F1) mice were interbred to produce the F2 inter-cross generation. F2 inter-cross mice were analyzed by flow cytometry to identify mice with the Jasmine phenotype (MHC-I low, few CD8 cells). DNA from 37 of these 'affected' mice was pooled and a panel of PCR markers spanning the 19 autosomal mouse chromosomes was tested for linkage with the phenotype. Linkage was observed to the H-2 I-E α locus at 34.48 Mb on chromosome 17. When tested individually, 37 out of 37 affected F2 mice from the cross were homozygous for the C57Bl/6 (H-2^b) version of this marker, indicating very strong linkage to this region.

The region around 18.7 cM on chromosome 17 is very gene rich, and contains MHC genes as well as genes involved in antigen presentation. It was unlikely that the phenotype was caused by mutations in MHC-I genes themselves, as three separate mutations would be required to inactivate all three mouse MHC-I genes (H-2K, H-2D and H-2L), thus preventing CD8T cell maturation. The two most likely candidate genes in the following region: Tap1, at 34.33 Mb and Tap2, at 34.35 Mb were therefore sequenced.

A single point mutation was detected in TAP2; an A to C transversion in exon 5, resulting in a threonine to proline substitution at position 293 in the TAP2 protein (Figure 3). Alignment of human and mouse TAP1 and TAP2 proteins reveals that the mutated threonine is present in all four proteins (Figure 3), consistent with an important role in TAP function.

Expression of TAP1 and TAP2

To determine whether the point mutation in the TAP2 gene affected mRNA stability, real time RT-PCR was carried out on thymocytes from C57Bl/6 and Jasmine mice. As shown in Figure 4a, TAP2 mRNA levels were equal in the two mouse strains. Western blotting experiments were therefore carried out to assess whether TAP2 protein levels are affected in Jasmine mice. As shown in Figure 4b, both TAP2 and TAP1 protein levels are substantially decreased in Jasmine mice, with TAP2 levels more profoundly affected than TAP1.

Figure 1 Diminished proportion of CD8⁺ lymphocytes in the thymus and the spleen of Jasmine mice. (a) Thymocytes and (b) Splenocytes from wild type, jasmine and $\beta_2m^{-/-}$ at 6–8 weeks of age. Left panels show representative flow cytometry plots from cells stained with anti-CD4 and anti-CD8, and pre-gated on lymphocytes by forward and side scatter. Splenocytes were depleted of erythrocytes before staining. Right panels show the average percentages of CD4 and CD8 cells from five mice (thymus) or three mice (spleen). Error bars represent standard error. Significant differences in CD8 are indicated (*= <0.05 , ***= <0.005 , Student's *t*-test, two-tailed).

Figure 2 Diminished major histocompatibility complex (MHC)-I expression in the thymus and the spleen of Jasmine mice. (a) Thymocytes and (b) Splenocytes from wild type, Jasmine and $\beta_2m^{-/-}$ mice at 6–8 weeks of age. Panels show representative flow cytometric histograms from cells stained with anti-H-2D^b (left) and anti-H-2K^b (middle) and the average of the mean fluorescence intensities (MFI) (right). Right panels show the average MFI from five mice (thymus) or three mice (spleen). Error bars represent standard error. Significant differences are indicated (*= <0.05 , ***= <0.007 , Student's *t*-test, two-tailed). Histograms are pre-gated on lymphocytes using forward and side scatter. Splenocytes were depleted of erythrocytes before staining.

Figure 3 The transporter associated with antigen processing (TAP)2 mutation in Jasmine affects a conserved residue. The mutated nucleotide and the resulting amino acid substitution are indicated. The protein sequences for mouse and human TAP1 and TAP2 were aligned using ClustalW software (<http://www.ebi.ac.uk/Tools/clustalw/>). Amino acids shaded in black are conserved in all four sequences, bolded and boxed amino acids are conserved in three out of the four sequences. The predicted sixth and seventh transmembrane helices of TAP2 are indicated by grey cylinders.

Identification of Rose (TAP1) mutation

At the time of submission of this manuscript, a second ethyl nitroso urea-induced TAP mutant strain, Rose, was identified. Rose has an A to T substitution in exon 10 of TAP 1 leading to an aspartic acid

to valine substitution in position 643. Again, the substitution affects an amino acid conserved in both human and mouse TAP1 and TAP2, but this time in the cytosolic Walker B motif, which is required for nucleotide binding. CD8 T-cell quantification in peripheral blood,

Figure 4 Transporter associated with antigen processing (TAP)2 protein levels are diminished in Jasmine mice. **(a)** Real time RT-PCR for TAP2 mRNA was carried out on RNA extracted from total splenocytes from wild type and Jasmine mice. TAP2 mRNA expression was normalized across the two strains to β -actin expression. **(b)** Western blotting carried out on splenocytes from wild type and Jasmine mice for TAP2 and TAP1, as well as β -actin, which was used as a loading control. Band density measurements (bold) and s.d. (in brackets) are shown on the right of the blot. Duplicate gels were run for each of the antibodies used. The lysate from 5×10^6 splenocytes was resolved per lane on a 12% SDS-PAGE gel. Shown are the results of four out of nine wild type–jasmine pairs analyzed.

MHC-I expression, and location of the mutation are shown in Figure 5.

Exogenous peptide rescues MHC-I surface expression

If the only defect in Jasmine and Rose mice is in the transport of antigenic peptides into the ER, the rest of the MHC-I antigen presentation pathway should be intact. This was first shown for TAP-deficient cell lines in a series of classic experiments, where exogenously added MHC-I-binding peptides were used to partially rescue surface MHC-I expression.^{32–34} Using a similar method, splenocytes from Jasmine and Rose mice were cultured with an H-2K^b-binding peptide (SSIEFARL from glycoprotein B of herpes simplex virus) and levels of surface H-2K^b and D^b measured by flow cytometry (Figure 6). As predicted for cells with a sole defect in TAP activity, the addition of SSIEFARL peptide to cells from both Jasmine and Rose mice partially restored MHC-I expression in an allele-specific manner.

DISCUSSION

In this paper we characterize the Jasmine mouse strain, which has a threonine to proline substitution at position 293 of TAP2, and also report the discovery of the Rose mouse strain, which has an aspartic acid to valine substitution at position 643 of TAP1.

Phenotype

In both cases the point mutations cause functional inactivation of TAP-dependent peptide transport, as evidenced by the severe reduction of MHC-I surface expression seen in the mice (Figures 2 and 5). The striking decrease in CD8⁺ T-cell numbers (Figures 1 and 5) would be predicted for a defect in a protein that is involved in MHC-I antigen presentation, as the lack of MHC-I would impede positive selection of CD8⁺ cells. This phenomenon is also observed in TAP1^{−/−} mice²⁰ and $\beta_2m^{-/-}$ mice,²⁴ where cells which would otherwise become CD8 SP cells are diverted into a ‘death by neglect’ pathway due to their

failure to interact with an appropriate peptide–MHC-I complex. Thus, we can conclude that the levels of MHC-I in both Jasmine and Rose mice are not sufficient to efficiently present peptides to CD8 T cells *in vivo*. The residual CD8⁺ T cells are likely to be either natural killer or $\gamma\delta$ T cells expressing CD8, or $\alpha\beta$ CD8⁺ T cells selected on the few remaining MHC-I molecules. Although the histograms in Figures 2 and 5 show that MHC-I levels in Jasmine mice are extremely low, they may not be completely absent. TAP-independent peptides such as those derived from signal sequences of secreted or membrane bound proteins,³⁵ or those generated from ER-specific degradation of proteins³⁶ are likely to be presented (albeit at a very low level).

Location of mutations

The mutation in Rose is located in the Walker B motif that forms a part of the nucleotide-binding domain of TAP1. The aspartic acid mutated in this strain is not only conserved between human, mouse chicken and shark TAP proteins, but is also seen in the nucleotide binding domain of the human cystic fibrosis transmembrane conductance regulator protein and other members of the ATP-binding cassette protein family.³⁷ This conservation would indicate an essential function for this residue. Although a number of studies^{37–39} have investigated the importance of the aspartic acid residue next to the one mutated in the Rose strains, no studies have been done so far to investigate the effect of mutations in residue 643 on the structure or function of TAP1.

The Jasmine mutation is located in the transmembrane domain of TAP2. Determining the membrane topology of TAP molecules has been challenging because of the lack of structural information on similar ATP-binding cassette transporters, and to the large number of transmembrane domains which preclude crystallization. Several different models for TAP1 and TAP2 have therefore been proposed.^{6,15,40–42} Of these, the most recent model,⁶ which was validated experimentally in a functional TAP dimer by probing the accessibility of cysteines introduced into predicted loops, is likely to be the most

Figure 5 Identification of the Rose strain. (a) Rose mice were identified owing to a greatly reduced proportion of CD8⁺ lymphocytes in peripheral blood. Flow cytometry plots show peripheral blood lymphocytes (gated by forward and side scatter) labelled with anti-CD8 and anti-CD4. (b) Panel shows a representative flow cytometric histogram from blood lymphocytes stained with anti-H-2K^b. (c) The mutated nucleotide and the resulting amino acid substitution are indicated. The protein sequences for mouse and human transporter associated with antigen processing (TAP)1 and TAP2 were aligned using ClustalW software (<http://www.ebi.ac.uk/Tools/clustalw/>). Amino acids shaded in black are conserved in all four sequences, bolded and boxed amino acids are conserved in three out of the four sequences. Dots represent amino acids not present in TAP2.

accurate. According to this model, threonine293 is located in a very short loop on the ER side of TAP2. This loop connects TM helices 6 and 7 (Figure 3) which are two of the six C-terminal transmembrane helices of TAP2, which, along with the six C-terminal helices of TAP1, are thought to form the core domain of the TAP transporter responsible for binding and translocating peptide across the ER membrane into the ER lumen.⁶

There are no previous mutagenesis studies in which a point mutation (or any deletions encompassing this amino acid) has been introduced into this region of TAP2.

It is likely that the mutation in Jasmine compromises the correct folding of TAP2, as the amino acid substitution in Jasmine represents a change from an uncharged polar to a non-polar amino acid with a cyclic side chain, which may have profound consequences for the local structure of the TAP2 molecule. All proteins to be inserted into the ER, whether soluble or membrane bound, undergo a highly regulated process of quality control, with mutated, incorrectly folded or truncated proteins, or proteins that fail to oligomerize, subject to rapid degradation.⁴³

Effect of lack of TAP2 on the stability of TAP1

Jasmine mice show substantially decreased levels of both TAP2 and TAP1 proteins (Figure 4b). This implies that the stability of TAP1

protein is dependent on TAP2. The reverse has previously been shown in a human cancer cell line,⁴⁴ where a TAP1 mutation results in no detectable protein expression of either TAP1 or TAP2. Our results are not consistent with the work of Keusekotten *et al*,⁴⁵ who have found that TAP 1 expressed alone is stable, but that TAP2 expressed alone degrades rapidly, thus concluding that the newly synthesized TAP2 assembles with the pre-existing TAP1. Their study, however, used rat TAP1 and TAP2 proteins expressed in a human TAP-deficient cell line, thus the difference is likely to be species specific, or alternatively may reflect the chimeric system they used.

Conclusion

The Jasmine mouse characterized in this study represents the first available mouse model with a deficiency in TAP2 function. Although two TAP1 knockout mouse strains are currently available,^{19,20} the Rose strain described in this paper harbours a single point mutation in TAP1. Neither of the amino acids affected by the mutations have previously been shown to affect TAP function, thus this study identifies new residues important for TAP function. The Jasmine and Rose strains should therefore be useful to the immunological community in further characterizing TAP function, and as useful models for Bare Lymphocyte syndrome. Finally, we identify a previously undescribed role for mouse TAP2 in stabilizing TAP1 protein.

Figure 6 Rescue of surface major histocompatibility complex (MHC)-I by the addition of peptides. Splenocytes from Jasmine (a) and Rose (b) mice were cultured for 2 h in the presence of 1×10^{-6} M synthetic SSIEFARL peptide (solid line) or medium alone (shaded) and analyzed for surface expression of H-2K^b (left plots) and D^b (right plots) by flow cytometry. Splenocytes from C57Bl/6 mice (dashed line) were included as positive controls. This experiment was independently replicated with a similar result.

METHODS

Development of mouse strains

Male C57Bl/6 mice were treated with ethyl nitroso urea, as previously described.³¹ Mice were screened by flow cytometry of blood for CD4, CD8 and CD44. Outlying mice or their littermates were bred to confirm heritability of the trait, and to establish each strain. The entire work was approved by the Australian National University Animal Ethics Committee, and carried out in accordance with institutional guidelines.

Knockout mice

β 2-Microglobulin knockout mice²³ were a kind gift from Professor Len Harrison (WEHI).

Mapping

The Jasmine mutation was mapped using NOD.H2k as the outcross strain, as previously described,³¹ and identified by sequencing genomic and cDNA. For the Rose strain, direct sequencing of the genes for TAP1 and TAP2 was carried out without a previous mapping cross.

Flow cytometry

The blood and spleen samples, depleted of erythrocytes by ammonium chloride lysis (10 mM KHCO₃, 150 mM NH₄Cl, 0.1 mM EDTA, pH 8.0), were stained with fluorochrome-conjugated antibodies (BD Biosciences, San Jose, CA, USA) in PBS/2% fetal bovine serum. Samples were collected on an LSRII flow cytometer (BD Biosciences). Data were analysed using FloJo software (Treestar, Ashland, OR, USA).

Real time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNA-free Turbo DNase (Ambion, Austin, TX, USA). First strand cDNA synthesis was carried out with Oligo-dT using Superscript III reverse transcriptase (Invitrogen). PCRs were carried out with Platinum SYBR Green qPCR SuperMix (Invitrogen) on a Corbett RotorGene 3000 thermocycler (Corbett Research, Mortlake, NSW, Australia).

Immunoblotting

Erythrocyte-depleted spleen cells (5×10^6 cells per lane) were lysed in buffer containing 1% Nonidet P-40, Tris-buffered saline (50 mM NaCl, 150 mM Tris-

HCl, pH 7.5) and complete protease inhibitor cocktail (Sigma, Castle Hill, NSW, Australia) for 60 min at 4°C and then centrifuged for 10 min at 12 000 g. Samples were resolved on 7.5% SDS-PAGE gels. Proteins were transferred to PVDF membranes, which were blocked overnight at 4°C in 0.2% iBlock (Applied Biosystems, Foster City, CA, USA) in Tris-buffered saline. Membranes were incubated with anti-TAP1, anti-TAP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti- β -actin antibodies (Abcam, Cambridge, MA, USA), followed by secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Bands were visualized with SuperSignal West Pico Chemiluminescent Substrate solution (Pierce, Rockford, IL, USA).

Exogenous addition of peptide

Erythrocyte-depleted splenocytes were incubated for 2–3 h at 37°C in Dulbecco's modified Eagle's medium with 1×10^{-6} M synthetic SSIEFARL peptide (Genscript, Piscataway, NJ, USA) and no foetal bovine serum. After this time, cells were stained with anti-H-2K^b-FITC (clone AF6-88.5) and anti-H-2D^b-PE (cloneKH95) in phosphate-buffered saline with 2% foetal bovine serum on ice.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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