Immunology 2002 **106** 182–189

Interactions formed by individually expressed TAP1 and TAP2 polypeptide subunits

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SUMMARY

The transporter associated with antigen processing (TAP) supplies peptides into the lumen of the endoplasmic reticulum (ER) for binding by major histocompatibility complex (MHC) class I molecules. TAP comprises two polypeptides, TAP1 and TAP2, each a 'half-transporter' encoding a transmembrane domain and a nucleotide-binding domain. Immunoprecipitation of rat TAP1 and TAP2 expressed individually in the human TAP-deficient cell line, T2, revealed that both bound the endogenously expressed HLA-A2 and -B51 class I molecules. Using HLA-encoding recombinant vaccinia viruses HLA-A*2501, -B*2704, -B*3501 and -B*4402, alleles also associated with both TAP1 and TAP2. Thus, TAP1 and TAP2 do not appear to differ in their ability to interact with MHC class I alleles. Single TAP polypeptide subunits also formed MHC class I peptide-loading complexes, and their nucleotide-binding domains retained the ability to interact with ATP, and may permit the release of peptide-loaded MHC class I molecules in the absence of a peptide transport cycle. It is also demonstrated by chemical cross-linking that TAP2, but not TAP1, has the ability to form a homodimer complex both in whole cells and in detergent lysates. Together these data indicate that single TAP polypeptide subunits possess many of the features of the TAP heterodimer, demonstrating them to be useful models in the study of ATP-binding cassette (ABC) transporters.

INTRODUCTION

Major histocompatibility complex (MHC) class I molecules require peptide for stable cell-surface expression, acquiring relevant peptides during assembly within the endoplasmic reticulum (ER). Such peptides, generated mostly by the activity of the cytosolic proteasome complex, are translocated in an ATP-dependent manner into the ER by an ATP-binding cassette (ABC) transporter, termed transporter associated with antigen processing (TAP).[1–3] This ER-located member of the ABC transporter[4] family comprises a heterodimer of TAP1 and TAP2 polypeptides, each encoding an N-terminal transmembrane domain (TMD) with multiple transmembrane spanning regions, and a C-terminal region encoding a nucleotide-binding domain (NBD). TAP is crucial in the assembly process of MHC class I molecules: in its absence MHC class I assembly is severely impaired. Furthermore, it can act as a hub where up to four non-peptide-loaded class I molecules, with associated accessory molecules, sit awaiting suitable peptides.[5–9]

The peptide transport specificity of TAP has been studied extensively in several species[2,10] and initial studies have started to identify the peptide-binding site.[11–13] The peptide transport process is strictly ATP dependent.[3] However, the mechanism by which peptide-loaded class I molecules dissociate from TAP is less clear. Recent data suggest the transmission of a signal from the NBD, allowing release of class I molecules, thus linking the active transport cycle of TAP to the release of peptide-loaded class I molecules.[14–16]

Most studies of TAP investigate the TAP heterodimer, however, and little is known about the behaviour of

Received 28 November 2001; revised 6 February 2002; accepted 11 February 2002.

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individual TAP1 or TAP2 polypeptide subunits. Although they are not active in peptide transport,[17] we have previously reported that both single TAP1 and TAP2 molecules retain the ability to interact with MHC class I molecules and chaperones.[18] Thus, they represent a simplified model for using to study some of the interactions that TAP can undertake. The data we present here demonstrates that single TAP polypeptide subunits interact with a wide variety of MHC molecules and retain features comparable to when present in the heterodimeric TAP complex. Single TAP polypeptide subunits therefore represent useful models to assist in our understanding of the TAP complex and ABC transporters in general.

MATERIALS AND METHODS

Cell lines and antibodies

The human lymphoblastoid cell line T2, transfected with rat TAP1 and rat TAP2 individually and together, was maintained in RPMI-1640 supplemented with 5% fetal calf serum (FCS) and 1·0 mg/ml G418, as required.[17,18] Antisera recognizing rat TAP1 and rat TAP2 have been described previously.[18] The monoclonal antibody (mAb) W6/32 recognizes human leucocyte antigen (HLA)-A, -B and -C molecules. Antiserum recognizing calreticulin was obtained from Stressgen (AMS Biotechnology Ltd., Abingdon, UK), antiserum recognizing ERp57 was a gift from Dr Tom Wileman (Institute for Animal Health, Pirbright, UK) and antiserum recognizing human tapasin was a gift from Dr Paul Lehner (University of Cambridge, Cambridge, UK). mAb MAC 394, recognizing rat TAP2, was a gift from Dr M. Knittler and Dr J. Howard (University of Cologne, Cologne, Germany). Antisera D90 (anti-rat TAP1) and 116 (anti-rat TAP2) were also used.[15,18]

Metabolic labelling and immunoprecipitation

Methionine- and cysteine-starved cells (30 min at 37°) were labelled with 3.7MBq of [^{35}S]Trans label (ICN Pharmaceuticals Ltd., Basingstoke, UK) for 15 min, and either lysed immediately in digitonin lysis buffer [0·5% digitonin, 150 mM Nacl, 10 mM Tris, pH 7·5, 5 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF)], or returned to normal medium for chase times as indicated prior to lysis. Immunoprecipitations were performed as previously described.[18] For peptide stabilization of TAP-associated class I MHC molecules, TAP polypeptides were isolated as described above, then incubated with 50 μM of HLA-A2-binding peptide (LLDVPTAAV)[15] on ice for 1 hr, then diluted in lysis buffer containing 1% Triton-X-100 instead of digitonin, and incubated at 37° for 1 hr. Peptide-loaded class I molecules were then isolated with antibody W6/32.

Large-scale purifications of TAP were performed using $\approx 2 \times 10^8$ cells lysed in digitonin buffer, as described above. Gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich, Poole, UK).

Isolation of TAPs with ATP-agarose beads (Sigma) were performed as per the immunoprecipitation protocols outlined above.[18] Cross-linking of TAP with 8-azido-adenosine 5′[α-^{32}P]triphosphate (Affinity Labelling Technology, Lexington, KY) was performed as described previously.[19] Briefly, antibody-immobilized TAP polypeptides, using sheep anti-TAP1 or -TAP2, were incubated for 15 min on ice in a 30-μl volume of lysis buffer containing 1 μCi $8N_3$ATP, then exposed to ultraviolet (UV) irradiation at 365 nm for 10 min on ice. Unlabelled ATP was included at a concentration of 0·5 mM. Free $8N_3$ATP was removed by washes in lysis buffer.

Recombinant vaccinia viruses

Vaccinia viruses expressing HLA alleles B*3501 and B*2704 have been described previously.[20,21] Recombinant viruses expressing A*2501 and B*4402 were constructed using standard procedures.[22] Vaccinia infections were performed at a multiplicity of infection of 10 for 1 hr at 37°. Cells were then incubated in fresh RPMI-1640 at 37° for 2 hr to allow expression of HLA alleles, before metabolic labelling and immunoprecipitation as described above. Proteasome inhibitors lactacystin and *N*-acetyl-Leu-Leu-Nle-H (Affiniti Research, Exeter, UK) were included, where indicated, at 100 μM. Two-dimensional gel analysis was performed as described previously.[23]

Chemical cross-linking of TAP

Whole cells were resuspended in phosphate-buffered saline (PBS) on ice, and ethylene glycol bissuccinimidyl succinate (EGS; Sigma) was added to final concentrations of 1–3 mM. After incubation for 30 min on ice, the cells were pelleted and resuspended in lysis buffer containing Triton-X-100. For cross-linking of cell lysates, lysis buffers containing digitonin or Triton-X-100 were used. EGS was added to the lysate and the reaction terminated by addition of sample buffer and heating. The TAP-binding peptide, TNKTVARYV, was added at a final concentration of 20 μM for 30 min on ice prior to EGS cross-linking.

RESULTS

TAP1 and TAP2 polypeptide subunits expressed singly interact with multiple MHC class I alleles

We have previously reported that both rat TAP1 and rat TAP2, when expressed as single polypeptide subunits in the human TAP-deficient cell line T2, display the ability to interact with MHC class I molecules and the chaperones calreticulin and tapasin.[18] However, our original observations utilized one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and could not distinguish whether the two HLA alleles (A2 and B51) expressed by the cell line differed in their ability to interact with TAP1 or TAP2. We therefore repeated this original experiment by immunoprecipitating TAP1 and TAP2 from digitonin lysates of metabolically labelled cells, and analysed the samples by two-dimensional electrophoresis, with isoelectric focusing in the first dimension followed by SDS–PAGE. As shown in Fig. 1, spots identified as HLA-A2 and -B51 (using HLA-A and -B-specific antibodies; results not shown) were immunoprecipitated with W6/32, along with β_2-microglobulin. The same spots were

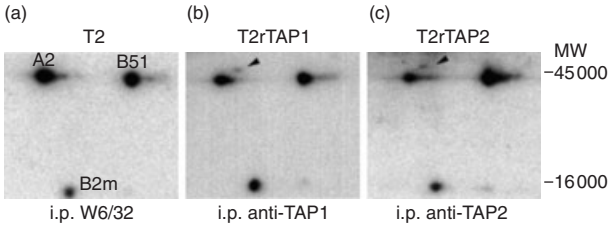

Figure 1. A2 and B51 alleles interact with transporter associated with antigen processing (TAP)1 and TAP2 expressed singly in T2 cells. T2 (a), T2rTAP1 (b) and T2rTAP2 (c) cells were metabolically radiolabelled and lysed in digitonin-containing buffers, followed by immunoprecipitation with antibodies recognizing human leucocyte antigen (HLA)-A, -B and -C, W6/32 (a), rat TAP1 (b) or rat TAP2 (c). Samples were analysed by two-dimensional electrophoresis. Only the portion of the gel containing the major histocompatibility complex (MHC) class I molecules is shown. The location of human tapasin is indicated by an arrowhead, and was confirmed by immunoprecipitation with anti-tapasin reagents and mass spectrometry (data not shown).

found in association with both TAP1 and TAP2 immunoprecipitations. Also visible in this image is human tapasin (arrowhead). This demonstrates that newly synthesized A2 and B51 alleles appear equally capable of association with TAP1 or TAP2.

We extended the above observations to investigate whether other HLA alleles demonstrated a similar ability to interact with TAP1 and TAP2. B*2704, B*4402, B*3501 and A*2501 alleles were expressed in the T2 transfectants using recombinant vaccinia constructs, and TAP immunoprecipitations were performed. As shown in Fig. 2, the vaccinia-expressed HLA alleles all shared the capability to interact with both TAP1 and TAP2. The one exception was A*2501, which appeared to interact weakly with TAP2. However, control W6/32 immunoprecipitations (performed on all the lysates; results not shown) indicated that the T2rTAP2 cell line repeatedly expressed A*2501 at lower levels than the T2rTAP1 cell line. In all cases the level of TAP association correlated with the W6/32-detected signal. We conclude from this data that TAP1 and TAP2 possess no intrinsic capacity to influence with which MHC class I molecule they associate, supporting the view that tapasin may be the most important determining factor in whether a class I molecule forms an association with TAP.[24]

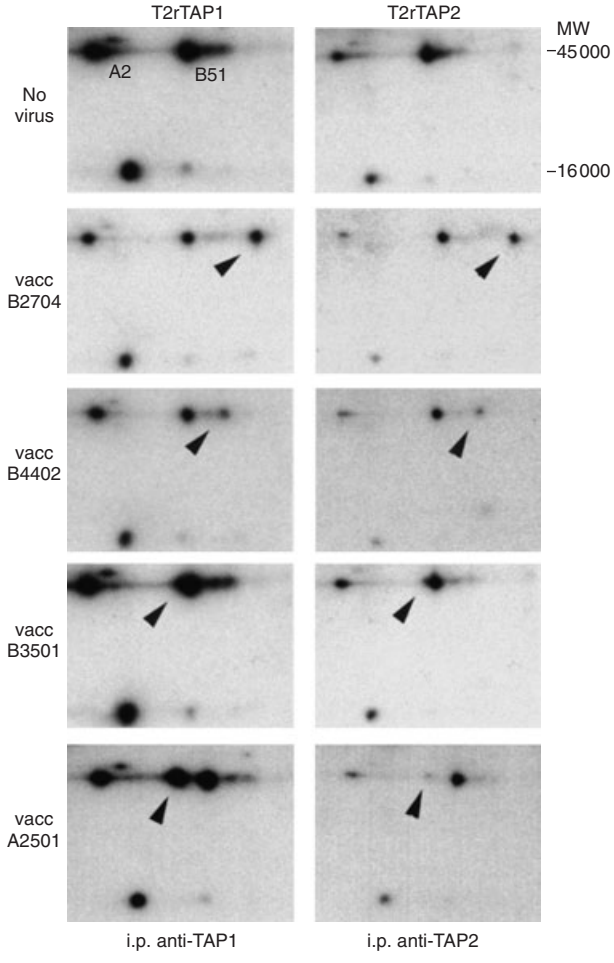

Figure 2. Multiple human leucocyte antigen (HLA) alleles interact with both transporter associated with antigen processing (TAP)1 and TAP2 subunits. T2rTAP1 and T2rTAP2 cells were infected with recombinant vaccinia viruses encoding HLA-B*2704, B*4402, B*3501 or A*2501 alleles. After metabolic radiolabelling and lysis in digitonin buffers, TAP1 and TAP2 were immunoprecipitated and samples analysed by two-dimensional electrophoresis. Only the portion of the gel containing the major histocompatibility complex (MHC) class I molecule is shown. Vaccinia (vacc)-encoded HLA alleles binding to TAP1 or TAP2 subunits are indicated by arrowheads. Immunoprecipitations of duplicate lysates were performed with W6/32 to control for possible differences in expression in the two cell lines (data not shown). Infections with HLA-negative viruses yielded results that were identical to the 'no virus' control.

MHC class I chaperone complexes in single TAP transfectants

In TAP-deficient cells such as T2, MHC class I molecules retain the ability to form complexes with chaperones such as calreticulin, ERp57 and tapasin.[9,25] We tested whether the presence of single TAP chains influenced the formation of these complexes. Digitonin lysates of metabolically labelled cells were immunoprecipitated with antibodies recognizing calreticulin, ERp57 and tapasin. As shown for T2rTAP1 cells in Fig. 3(a), complexes containing A2 and B51 MHC class I molecules were detected with each of the accessory molecules (arrowhead). Identical results were obtained in T2, T2rTAP2 and T2rTAP1 + 2 cells (results not shown), indicating that formation of these complexes may be relatively independent of the presence of TAP polypeptides.

Figure 3(a) demonstrates that immunoprecipitation of calreticulin, ERp57 or tapasin co-precipitates strong signals for MHC class I molecules, but only weak signals for the other chaperones. This may be a result of the short metabolic labelling times used in these experiments and suggests

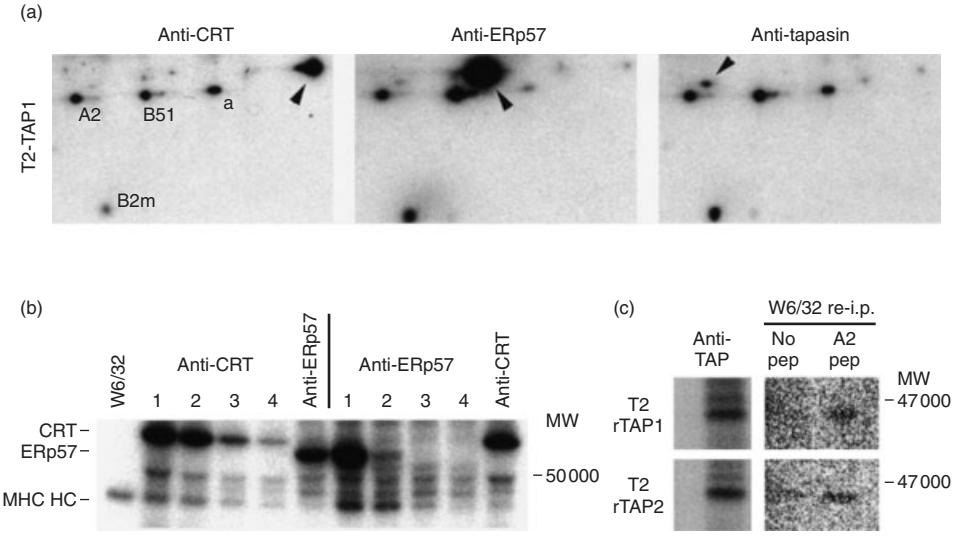

Figure 3. Formation of major histocompatibility complex (MHC) class I/chaperone complexes by transporter associated with antigen processing (TAP) subunits. (a) T2rTAP1 cells were metabolically radiolabelled, lysed in digitonin and immunoprecipitated with antibodies to calreticulin (CRT), ERp57 or tapasin. Arrowheads indicate the position of chaperones, as determined by mass spectrometry and immunoprecipitation in Triton-X-100 lysis buffers (not shown). Only the portion of the gels containing MHC class I and chaperones are shown. The position of a contaminant identified as actin is indicated by the small letter 'a'. (b) T2rTAP1 cells were metabolically radiolabelled, lysed in digitonin buffers and the lysate split in three portions. Lysates were immunoprecipitated with the monoclonal antibody W6/32 (first lane), or consecutively with anti-CRT or anti-ERp57 reagents, followed by anti-ERp57 or anti-CRT, respectively. Samples were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). (c) TAP1- and TAP2-associated A2 molecules are stabilized by peptide. T2rTAP1 and T2rTAP2 cells were metabolically radiolabelled, lysed in digitonin and split into three portions. After immunoprecipitation of TAP polypeptides, complexes were incubated with or without A2-binding peptide (LLDVPTAAV), dissociated from TAP and reimmunoprecipitated with W6/32, as described in the Materials and methods. Samples were analysed by SDS–PAGE.

that newly synthesized chaperones and class I MHC associate mostly with pre-existing pools of the other components of the peptide-loading complex. Alternatively it is possible that these other chaperones were not co-precipitated and that the data represent three different pools of class I MHC–chaperone complexes. To test this we performed several rounds of depletion of calreticulin from digitonin lysates, followed by immunoprecipitation of ERp57 (Fig. 3b). Essentially no MHC class I was recovered in association with ERp57 after calreticulin depletion. Similarly, after ERp57 depletion no MHC class I was detected in association with calreticulin. From this data we conclude that the majority of MHC class I–chaperone complexes in the ER exist as complexes that contain both calreticulin and ERp57, and that very few complexes normally exist with only one chaperone attached.

HLA-A2 dissociates from single TAP polypeptides

The data shown in Fig. 1 indicate that A2 and B51 alleles interact strongly with TAP polypeptides during brief metabolic labelling. A strikingly different result was obtained when single TAP polypeptides were immunoisolated in larger quantities for proteomic identification of components. TAP1, TAP2 and the TAP heterodimer were immunoisolated from digitonin lysates of $\approx 2 \times 10^8$ cells, and the resulting two-dimensional gels were stained with Coomassie Blue (Fig. 4a). In the control (T2), sample spots contributed by the anti-TAP antibodies alone (labelled with asterisks) and actin (labelled 'a') were visible. The T2rTAP1 sample showed the additional presence of ERp57, calreticulin, tapasin, HLA-B51 and β_2-microglobulin. However, in contrast to our data in Fig. 1, we failed to detect significant quantities of HLA-A2. Similarly, almost no A2 association was found in purifications from T2rTAP2 and T2rTAP1 + 2 cells. Pulse–chase analysis was therefore performed to determine the rate of loss of A2 from single TAP polypeptides (Fig. 4b). Over a chase period of 1 hr, 90% of A2 and 25% of B51 molecules dissociated from the TAP heterodimer in T2rTAP1 + 2 cells (A2 data additionally shown in Fig. 4c), presumably as a result of active peptide supply from the cytosol via TAP. With single TAP polypeptides, 51% of A2 dissociated, whereas essentially no B51 was lost. These dissociation kinetics could explain why reduced steady-state levels of A2 association with TAP were detected (Fig. 4a). However, in the absence of peptide transport in the single TAP transfectant lines, we could not invoke the supply of cytosolic peptides to explain A2 dissociation. HLA-A2 molecules, however, are known to be able to bind and complete assembly with hydrophobic peptides derived from signal sequences.[26] To test whether signal peptides in the ER

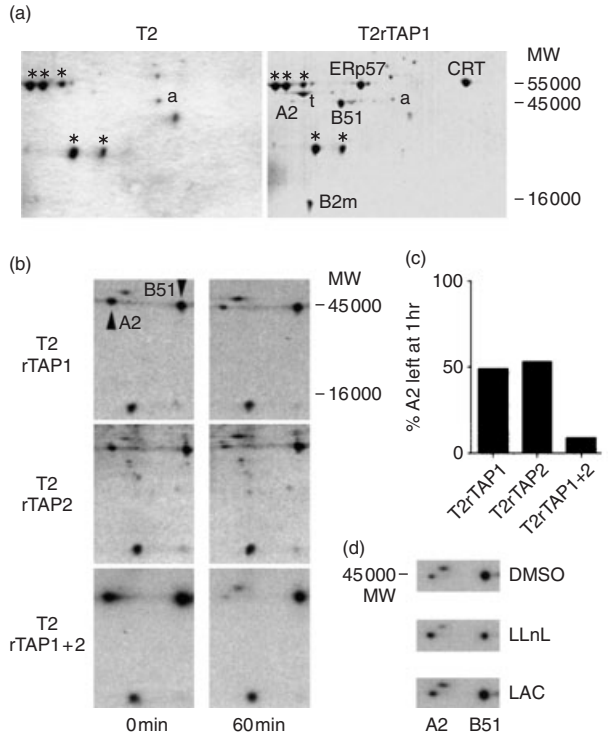

Figure 4. Human leucocyte antigen (HLA)-A2 molecules dissociate from single transporter associated with antigen processing (TAP) subunits. (a) T2 and T2rTAP1 cells were lysed in digitonin and immunoprecipitated with antibodies to TAP1. Samples were analysed by two-dimensional electrophoresis and gels stained with Coomassie Blue. Anti-TAP1 antibody H- and L-chains are indicated by asterisks. Actin is shown as a small letter 'a'. The identities of major histocompatibility complex (MHC) class I and chaperones were confirmed by mass spectrometry (except for β_2-microglobulin, which was confirmed by immunoprecipitation). TAP subunits do not resolve accurately during the isoelectric focusing step and do not appear on these gels. (b) T2rTAP1, T2rTAP2 and T2rTAP1+2 cells were metabolically radiolabelled and the samples either lysed immediately or chased for 1 hr and then lysed, followed by immunoprecipitation of TAPs. Only the major histocompatibility complex (MHC) class I region of the gel is shown. (c) Densitometric analysis of the TAP-associated A2 signal from (b). (d) T2rTAP1 cells were metabolically radiolabelled in the presence of protease inhibitors LLnL, lactacystin or control dimethylsulphoxide (DMSO). Cells were chased for 90 min, lysed in digitonin and TAP1 immunoprecipitated. Samples were analysed by two-dimensional electrophoresis. Only the region of the gels containing MHC class I heavy chain (and tapasin) spots are shown.

could lead to A2 dissociation from TAP, pulse–chase experiments were performed in the presence of the protease inhibitor LLnL, which has been shown previously to inhibit A2 assembly in the ER in TAP-deficient cells.[27] As shown in Fig. 4(d), A2 molecules were retained in association with TAP1 in the presence of LLnL, but not the more specific proteasome inhibitor, lactacystin, after a 90-min chase. With respect to A2 association levels at the start of the chase period (results not shown) control dimethylsulphoxide (DMSO)- and lactacystin-treated cells retained 37% and 51% A2 association, respectively, whilst LLnL-treated cells retained 93% A2. This suggests that the dissociation of A2 from individual TAP polypeptide subunits is induced by the association of peptides produced in the ER, and that individual TAP chains permit peptide-induced class I dissociation.

To further determine whether the A2 molecules associated with individual TAP1 and TAP2 polypeptide subunits were in a peptide-receptive state, we added the A2-binding peptide, LLDVPTAAV, to single TAPs isolated from digitonin lysates. A2 molecules were then dissociated from TAP subunits by addition of Triton-X-100, and non-peptide-loaded A2 molecules were denatured by incubation at 37°. The recovery of W6/32-reactive class I complexes from peptide-treated samples, but not from untreated controls, indicated that the class I MHC complexes associated with single TAP subunits were capable of binding peptides (Fig. 3c). Combined with the observations above, our data indicate that single TAP subunits associate with peptide-receptive MHC class I–chaperone complexes, and that upon the supply of appropriate peptides, may also permit the release of assembled MHC class I molecules.

Single TAP subunits retain ATP-binding sites

Disruption of the ATP-binding site of TAP transporters by mutagenesis is known to inhibit peptide-induced MHC class I dissociation.[14] We therefore determined whether single TAP polypeptides expressed in T2 cells retained the ability to interact with ATP, reasoning that our observations on A2 loss from single TAP subunits suggest that ATP binding should be retained. Lysates from T2, T2rTAP1, T2rTAP2 and T2rTAP1+2 cells were incubated with ATP-agarose beads and the resulting complexes were analysed by SDS–PAGE and Western blotting. Both single TAP1 and TAP2 subunits retained the ability to interact with ATP, albeit at reduced capacity when compared to the TAP heterodimer (Fig. 5a). Addition of excess ATP inhibited the interaction with ATP-agarose beads (Fig. 5b), and ATP binding was also demonstrated by cross-linking to radiolabelled $8N_3ATP$ (Fig. 5c). These data indicate that single TAP subunits do retain, at least in part, the ability to bind ATP.

TAP2 subunits form homodimeric complexes

Homodimerization of TAP subunits has been suggested to occur through both the TMDs and NBDs.[28,29] We examined whether the single TAP subunits expressed in T2 cells could form homodimers by chemical cross-linking with EGS, which has previously been shown to form a high-molecular-mass cross-linked TAP product.[30] The TAP heterodimer in whole T2rTAP1+2 cells was cross-linked with EGS into a product of ≈200 000 molecular weight (MW), as revealed by immunoblotting with anti-TAP2 (Fig. 6a) and anti-TAP1 antibodies (data not shown). No cross-linked product was detected in T2rTAP1 cells, although cross-linked products of similar MW were detected in T2rTAP2 cells, together with additional minor

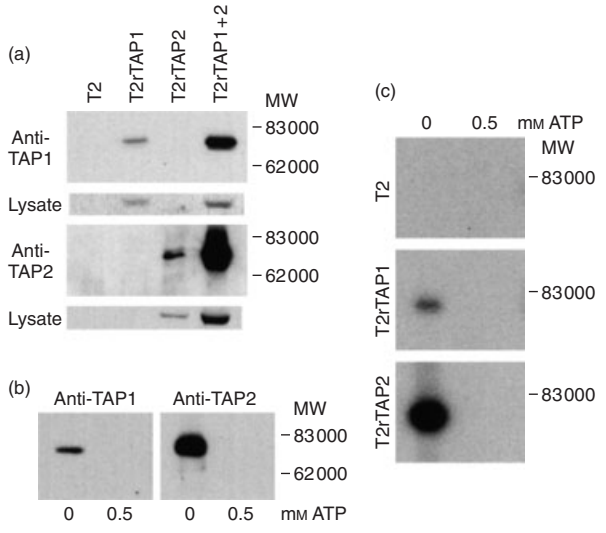

Figure 5. Single transporter associated with antigen processing (TAP) subunits bind ATP. (a) T2, T2rTAP1, T2rTAP2 and T1rTAP1 + 2 cells were lysed in Triton-X-100-containing buffer and incubated with ATP-agarose beads. Washed complexes were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting for TAP1 and TAP2. Control lysates are shown below each ATP-agarose gel. (b) The ATP-agarose isolation experiment from (a) was repeated with T2rTAP1 and T2rTAP2 cell lysates supplemented with or without 0·5 mM ATP. (c) Single TAP subunits were immunoprecipitated from T2rTAP1, T2rTAP2 or control T2 cells, and the immune complexes cross-linked to 8-azidoadenosine 5′[α-^{32}P]triphosphate. Sample were analysed by SDS–PAGE.

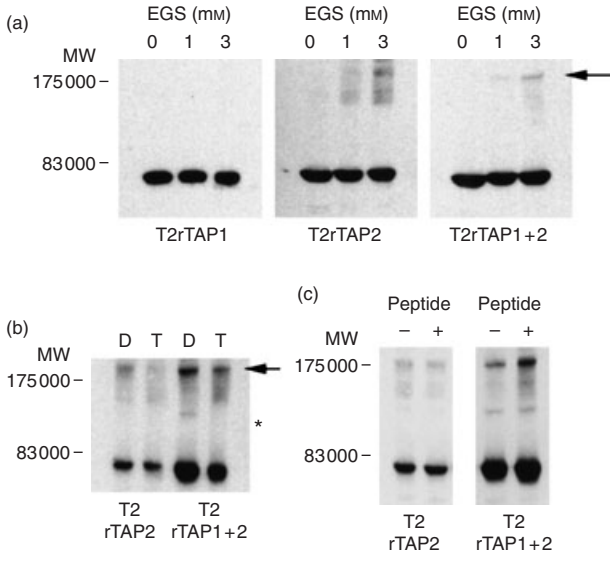

Figure 6. Chemical cross-linking reveals a transporter associated with antigen processing (TAP)2 homodimer. (a) Intact T2rTAP1, T2rTAP2 and T2rTAP1 + 2 cells were incubated with increasing concentrations of ethylene glycol bissuccinimidyl succinate (EGS). Cells were then lysed in Triton-X-100-containing buffer and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting for TAP1 or TAP2. The arrow indicates the formation of a cross-linked TAP product. (b) T2rTAP2 and T2rTAP1 + 2 cells were lysed in digitonin (D) or Triton-X-100 (T) buffers prior to cross-linking with EGS. Samples were than analysed by SDS–PAGE and Western blotting for TAP2. An asterisk indicates the position of an additional, as yet unidentified, cross-linked product. (c) Digitonin lysates of T2rTAP2 and T2rTAP1 + 2 cells were incubated with or without 20 μM of TAP-binding peptide (TNKTVARYV) prior to EGS cross-linking. Samples were analysed by SDS–PAGE and Western blotting for TAP2.

products. We then investigated whether the TAP2 homodimeric structure could be detected in detergent cell lysates. As shown in Fig. 6(b), whereas the TAP1 + 2 heterodimer could be cross-linked in lysates containing digitonin and Triton-X-100, TAP2 homodimers were only detected in digitonin. This suggests that TAP2 polypeptide chains interact more weakly with each other than with TAP1 chains, thus favouring the formation of heterodimers in wild-type cells. Intriguingly, in digitonin lysates of T2rTAP1 + 2 cells, we also detected another cross-linked product of smaller MW than cross-linked TAP (indicated by an asterisk in Fig. 6b). This may represent EGS cross-linking of another component of the TAP–MHC peptide-loading complex (preserved in digitonin, e.g. see Fig. 4a) to the TAP2 subunit. At present we have been unable to determine the identity of this extra band. Finally, we determined whether the cross-linking of TAP2 homodimers could be enhanced by the inclusion of a TAP-binding peptide. Previous work has demonstrated that a peptide-bound form of TAP has an increased ability to form a cross-linked structure in the presence of EGS.[30] Incubation of digitonin lysates, to preserve the TAP2 homodimer structure, with the peptide TNKTVARYV prior to EGS cross-linking, led to an observed increase in the TAP1 + 2 cross-linked product, but not the TAP2 homodimer (Fig. 6c).

DISCUSSION

TAP has been studied extensively and we know it to be an ER-resident, ATP-dependent peptide pump, with a promiscuous binding site formed combinatorially by intracellular loops of TAP1 and TAP2 subunits.[3,31–33] More recently the mechanisms which regulate TAP function have begun to be dissected, revealing complexity in the cycle of nucleotide hydrolysis and release of peptide-loaded MHC class I molecules.[14,16,34] In this study we set out to investigate the characteristics of single TAP subunits, about which relatively little are known in comparison to the TAP heterodimer.

We extended our original observations on the ability of single TAP1 and TAP2 subunits to interact with MHC class I molecules.[18] Multiple HLA alleles were found to be able to form interactions with both subunits. Thus neither TAP1 nor TAP2 seem capable of distinguishing or selecting what MHC class I molecule they are interacting with. This implies that tapasin bears responsibility for determining the success of the TAP–MHC interaction, and that inefficient interaction with TAP by some HLA alleles is caused

solely by weak MHC class I–tapasin interactions.[35,36] The binding site for MHC class I–tapasin complexes on TAP is not currently known; however, our data suggest that any potential site(s) on both TAP1 and TAP2 share similar features. We have been unable to verify the number of MHC class I complexes associated with each TAP subunit in this study because TAPs failed to resolve in the two-dimensional gels under conditions where MHC class I and chaperones did resolve. Nevertheless, our data suggest that class I MHC, calreticulin, ERp57 and tapasin are present at essentially equimolar ratios when associated with TAP.[8]

Our observations that HLA-A2 molecules have low steady-state levels of association with single TAP subunits were somewhat unexpected, based on our initial metabolic labelling and immunoprecipitation experiments. Mutagenesis of the nucleotide-binding sites on TAP1 and TAP2 has been shown to prevent both peptide transport and dissociation of MHC class I molecules.[14] We therefore expected a failure of MHC class I dissociation to occur in individual TAP1 and TAP2 subunits where no peptide transport occurs. Our observation, that LLnL (which can inhibit protease activity in the ER; see ref. 27) reduced A2 dissociation, suggests that the loading of ER-derived, probably signal sequence, peptides may be the cause of the A2 dissociation. The fact that single TAP subunits also retained some ability to bind ATP suggests that retention of an ATP-binding (though non-hydrolysing) NBD maintains a conformation of the whole TAP subunit, which permits the dissociation of MHC class I molecules upon loading with suitable peptide. Densitometry analysis of the results in Fig. 5, combined with other similar experiments (data not shown), suggest that the individually expressed TAP1 and TAP2 subunits can bind to ATP-agarose beads at between 40 and 70% of the level of the TAP heterodimer, although considerable variation occurs between experiments. Further detailed analysis would need to be performed to more accurately assess the ATP-binding capacity of the TAP1 and TAP2 subunits. Furthermore, the stability of the individual TAP chains appears to differ. In pulse–chase experiments of T2rTAP1 and T2rTAP2 cells, the TAP2 polypeptide is degraded more rapidly than TAP1 (S. J. Powis, unpublished). We are also currently investigating, by the use of ER-targeted minigene constructs and membrane-translocating 'trojan' carrier peptides,[37] whether we can also induce the dissociation of B51 molecules from individual TAP1 or TAP2 subunits.

The potential for homodimeric structures of TAP1 and TAP2 arises from the significant homology shared by these two polypeptides and the fact that some ABC transporters do function as homodimers. Homodimeric interactions have been detected during expression of truncated TAP NBDs[29] and in chimeric TAPs,[28] although not in the crystal structure of the NBD of TAP1.[38] In the experiments reported here we used the chemical cross-linker EGS, which can cross-link heterodimeric TAP, to show the formation of a TAP2 homodimer. We did not detect a similar TAP1 homodimer, although this does not preclude its existence. The optimal proximity of suitable residues for EGS cross-linking may be available in rat TAP2, but not in rat TAP1, homodimers.

The formation of the heterodimeric EGS cross-linked TAP product was enhanced by TAP-binding peptides, but a high proportion of TAP remains non-cross-linked.[30] This suggests that in the presence of peptide there is closer proximity of some regions of the TAP1 and TAP2 subunits, and that the cross-linking may occur during distinct stages of the TAP cycle. The failure of peptide to enhance TAP2 homodimer cross-linking indicates that similar events do not occur in this structure.

We have recently performed single-image particle analysis by electron microscopy of the TAP heterodimer and the TAP2 subunit on its own to determine a three-dimensional structure for TAP.[19] We did not detect homodimeric TAP2 structures during that study, probably because our immunoaffinity-based purifications were performed in lysates containing Triton-X-100 which, as described here, do not appear to support the interactions of homodimeric TAP2 post-lysis. However, the use of EGS does provide us now with a method by which we may be able to isolate and perform single-particle image analysis on the TAP2 homodimer.

Crystallographic determination of the structure of TAP has not yet been achieved, and it is only very recently that a whole ABC transporter, the homodimeric MsbA from *Escherichia coli*, has been crystallised and the structure determined to 4.5 Å resolution.[39] The data we present here, in conjunction with our preliminary analysis by single-particle imaging of TAP2,[19] suggests that single TAP subunits retain sufficient structural integrity and similarities to when present in the TAP heterodimer, to make them ideal substrates for crystallographic studies and also for mutagenic studies to identify tapasin-binding sites.

ACKNOWLEDGMENTS

S.J.P. is supported by a UK Medical Research Council (MRC) Senior Fellowship, and S.F. is supported by an MRC PhD studentship. E.S.P. was supported by a summer scholarship from the Wellcome Trust Biocentre, Dundee. We thank Nick Morrice for help with proteomics.

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