Detection of TAP Family Dimerizations by an *in Vivo* Assay in Mammalian Cells†

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Received April 29, 2004; Revised Manuscript Received September 3, 2004

ABSTRACT: The transporter associated with antigen presentation (TAP) is an ATP-binding cassette (ABC) protein which transports peptides for presentation to the immune system. TAP is composed of two half transporters, TAP1 (ABCB2) and TAP2 (ABCB3), which heterodimerize to function. In humans, the TAP family consists of TAP1, TAP2, and TAPL (ABCB9). While the TAP1−TAP2 complex is well characterized, TAPL's dimerization state and function are unknown. To identify interactions within the human TAP family, we adapted the dihydrofolate reductase protein-fragment complementation assay (DHFR PCA) to half ABC transporters. This assay has been shown to be suitable for the study of membrane-bound proteins *in vivo* [Remy, I., Wilson, I. A., and Michnick, S. W. (1999) *Science 283*, 990−993]. With this method, *in vivo* TAP1−TAP2 heterodimerization was confirmed, no homodimerizations were detected with TAP1 or TAP2, and TAPL did not show any interaction with TAP1 or TAP2. However, we found strong evidence that TAPL forms homodimers. These results provide evidence of a novel homomeric TAPL interaction and demonstrate that the DHFR PCA will be of general utility in studies of half ABC transporter interactions *in vivo*.

Perhaps the best characterized half ABC transporters are the ones which form the transporter associated with antigen presentation (TAP).[1] TAP translocates peptides from the cytoplasm into the lumen of the endoplasmic reticulum (ER), where they are assembled into major histocompatibility complex (MHC) class I molecules for presentation to the immune system (reviewed in ref *1*). This transporter is a heterodimer consisting of TAP1 (ABCB2) and TAP2 (ABCB3). Genetic evidence that TAP1 and TAP2 form heterodimers comes from studies of human mutant cell lines which found that expression of both genes was needed for antigen processing (*2*−*4*). Co-immunoprecipitation studies indicate that TAP1 and TAP2 are found as a complex in the ER membrane (*5*, *6*). This TAP heterodimer has been shown to function without any additional factor of the immune system (*7*, *8*). A direct physical interaction has also been demonstrated between TAP1 and TAP2 when photoreactive peptide analogues labeled both TAP1 and TAP2, suggesting that the peptide-binding site of TAP is formed by amino acids from both TAP1 and TAP2 (*9*). Cross-linking experiments and gel filtration analysis suggest that TAP1 and TAP2 form a functional heterodimer with a stoichiometry of 1:1 (*8*, *10*, *11*). Transmission electron microscopy has since confirmed that TAP1 and TAP2 form a single heterodimeric complex (*12*).

The third and final member of the TAP family is the TAP-like (TAPL, ABCB9) protein, which shares 38% and 40% amino acid sequence identity with TAP1 and TAP2, respectively. There is some debate over whether TAPL is localized to lysosomes or the endoplasmic reticulum, where TAP1 and TAP2 are localized (*13*, *14*). High expression of TAPL was found in testis, and moderate expression was found in brain, spinal cord, and thyroid (*13*). Staining patterns seen with anti-TAPL antibody indicate that it is expressed in the Sertoli cells of the seminiferous tubules (*13*), which form part of the blood−testis barrier separating spermatogonia from spermatocytes and spermatids. Although its function is unknown, TAPL's high degree of amino acid sequence identity with TAP1 and TAP2 suggests that it may be a peptide transporter and that it may dimerize with TAP1 or TAP2.

While it is well-known that TAP1 forms a heterodimer with TAP2, there is also some evidence that TAP1 may form homodimers (*15*, *16*). When rat TAP1 was introduced into murine small cell lung carcinoma cells, the cells gained the ability to be recognized by specific cytotoxic T-lymphocytes (CTLs) when infected with vesicular stomatitis virus (VSV) (*15*). Furthermore, the introduction of rat TAP1 caused VSV peptides to bind to putative lumenal ER proteins, suggesting

† This work was supported by research grants from the Canadian Institutes of Health Research (MOP 42560) and the National Cancer Institute of Canada (NCI 11410). V.L. is a Distinguished Scholar of the Michael Smith Foundation for Health Research.

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[1] Abbreviations: ABCB2, ATP-binding cassette subfamily B transporter 2; ABCB3, ATP-binding cassette subfamily B transporter 3; ABCB9, ATP-binding cassette subfamily B transporter 9; bp, base pairs; CHO, Chinese hamster ovary; CMV, cyto-megalo virus; CTL, cytotoxic T-lymphocyte; DHFR PCA, dihydrofolate reductase protein-fragment complementation assay; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HT, hypoxanthine and thymidine; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; TAP, transporter associated with antigen presentation; TAP1, transporter associated with antigen presentation 1, also known as ABCB2; TAP2, transporter associated with antigen presentation 2, also known as ABCB3; TAPL, TAP-like protein, also known as ABCB9; SDS, sodium dodecyl sulfate; UTR, untranslated region; VSV, vesicular stomatitis virus.

10.1021/bi0491245 CCC: $27.50 © 2004 American Chemical Society
Published on Web 10/16/2004

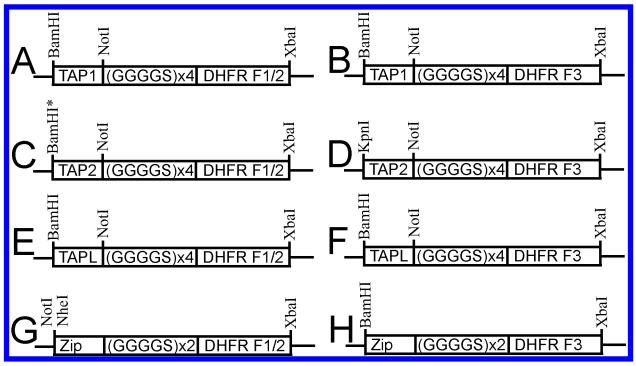

FIGURE 1: Maps of DHFR construct inserts. Constructs from left to right are (A) TAP1-L_{20}-F1/2, (B) TAP1-L_{20}-F3, (C) TAP2-L_{20}-F1/2, (D) TAP2-L_{20}-F3, (E) TAPL-L_{20}-F1/2, (F) TAPL-L_{20}-F3, (G) Zip-F1/2, and (H) Zip-F3. The flexible linker consists of repeating units of the amino acids GGGGS. All F1/2-containing contructs were in pcDNA3, and all F3 contructs were in pcDNA3.1/ZEO which had the *Nhe*I site removed. The asterisk indicates that a *Bgl*II site on the 5′ end of TAP2-L-F1/2 was used to clone into the *Bam*HI site of pcDNA3, thus eliminating both sites.

that they had been transported by TAP1 alone (*16*). Their finding that proper immune function could be restored by introduction of rat TAP1 alone led these authors to conclude that TAP1 forms homodimers.

In addition to the question of TAP1 homodimers, it is not known if TAP1 and/or TAP2 may interact with TAPL or if TAPL or TAP2 forms homodimers. While a TAP2 homodimer has been proposed (*17*), there is no conclusive biochemical evidence for such a pairing. As TAPL's dimerization state has not been examined, it is possible that it may interact with TAP1 or TAP2 or form homodimers. It may be that these transporters behave like white, brown, and scarlet in *Drosophila*, where white pairs with either brown or scarlet to transport different substrates (*18*−*21*). Therefore, there is a need to assay which interactions occur *in vivo* between all of the members of the TAP family.

To study interactions among TAP family members, we adapted the dihydrofolate reductase protein-fragment complementation assay (DHFR PCA) to this family. This assay was shown to be suitable for the study of membrane-bound proteins *in vivo* when it was used to detect ligand-induced conformation change in the erythropoietin receptor (*22*). The DHFR PCA is a direct way of determining if a physical interaction exists between two proteins. This method was chosen as it can detect protein−protein interactions embedded in various subcellular membranes regardless of which membranes they are in, can work in mammalian cells, and does not rely on indirect evidence of interaction. Using this approach, we sought to confirm, *in vivo*, the interaction between TAP1 and TAP2 and to assay for other interactions between TAP1, TAP2, and TAPL.

MATERIALS AND METHODS

Plasmids for DHFR PCA. TAP1 and TAP2 cDNAs were gifts from Dr. Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA). Leucine zipper DHFR contructs, designated Zip-F1/2 (for the N-terminal half of DHFR) and Zip-F3 (for the C-terminal half of DHFR) (*23*), were used to generate each of the DHFR contructs. TAP1-L_{20}-F1/2, TAP2-L_{20}-F1/2, and TAPL-L_{20}-F1/2 (Figure 1) were created by replacing the TAP stop codons with *Not*I restriction sites and cloning them in-frame with fragment F1/2 (from Zip-F1/2) with an extra 10 amino acid linker to bring the total linker length to 20 amino acids. TAP1-L_{20}-F3, TAP2-L_{20}-F3, and TAPL-L_{20}-F3 (Figure 1) were created in an analogous fashion by replacing the TAP stop codons with *Not*I restriction sites and cloning them in-frame with fragment F3 (from Zip-F3). All F1/2-containing contructs were in pcDNA3 [neomycin resistance gene, cyto-megalo virus (CMV) promoter, from Invitrogen, Burlington, Ontario, Canada], and all F3 contructs were in pcDNA3.1/ZEO (Zeocin resistance gene, CMV promoter, from Invitrogen). Primers used to add restriction sites were TAP1BH1 (5′-GCTAGGATCCATGGCTAGCTCTAGGTGTCCGC-TCCC-3′) and TAP1Nt (5′-GCTAGCGGCCGCTTCTG-GAGCATCTGCAGGAGCCTGCAC-3′) for TAP1-L_{20}-F1/2 and TAP1-L_{20}-F3, TAP2Bgl2 (5′-GCTAAGATCTATGCG-GCTCCCTGACCTGAGACCCTGG-3′) and TAP2Nt (5′-GCTAGCGGCCGCGTCCATCAGCCGCTGCTGAACCA-GGCG-3′) for TAP2-L_{20}-F1/2, TAP2KnI (5′-GCTAGGTAC-CATGCGGCTCCCTGACCTGAGACCCTGG-3′) and TAP2-Nt for TAP2-L_{20}-F3, and CLO15 (5′-GCTAGGATCCAT-GCGGCTGTGGAAGGCG-3′) and CLO13 (5′-GCTAGCG-GCCGCGGCCTTGTGACTGCCG-3′) for TAPL-L_{20}-F1/2 and TAPL-L_{20}-F3.

After restriction sites were added to TAP1, TAP2, and TAPL by polymerase chain reaction (PCR) and ligated into the pcDNA3 and pcDNA3.1/ZEO vectors, all the constructs were sequenced for verification. TAP1 and TAPL inserts were identical to published sequences, but TAP2-L_{20}-F3 contained the following changes: I → V at amino acid (AA) 378 and a silent mutation at AA 385; TAP2-L_{20}-F1,2 contained these two changes as well as a R → K at AA 661. These were considered acceptable because one gave the same amino acid and other two gave amino acids that are similar in size and character.

Cell Lines and Culture. The Chinese hamster ovary (CHO) cell line CHO DUKX-B11, a DHFR-deficient cell line originally characterized by L. Chasin (*24*, *25*), was maintained in minimum essential alpha medium with 292 mg/L L-glutamine, without ribonucleosides and deoxyribonucleosides (Invitrogen), and supplemented with 105 units of penicillin−streptomycin (Invitrogen)/mL, 105 μM sodium hypoxanthine, 16.8 μM thymidine [from hypoxanthine and thymidine (HT) supplement (Invitrogen)], and 10.5% dialyzed fetal bovine serum (FBS; Hyclone, Logan, UT). Nucleotide-free medium is identical to the above medium except that it does not contain the HT supplement.

BRE-169 (TAP1-deficient), STF1−169 (TAP2-deficient), and STF1−169/TAP1.2 (TAP2-complemented STF1−169) cell lines were all gifts from Dr. Henri de la Salle (Etablissement Français du Sang−Alsace, Strasbourg Cédex, France). Each line was cultured in Dulbecco's modified Eagle's medium with 4500 mg/mL glucose, 584 mg/mL L-glutamine, and 110 mg/L sodium pyruvate and pyridoxine hydrochloride (Invitrogen), supplemented with 105 units of penicillin−streptomycin (Invitrogen)/mL and 10.5% dialyzed fetal bovine serum (Hyclone).

All cells lines were grown at 37 °C in a 5% CO_2 atmosphere in a humidified incubator.

DHFR Survival Assay. To detect interactions between members of the TAP family, TAP genes are fused to complementary N- and C-terminal fragments (fragments F1/2

and F3). If the two half ABC transporters dimerize, they will induce the folding of F1/2 and F3 fragments into an active DHFR molecule. In DHFR-deficient cells, introduction of active DHFR will enable the cells to survive in nucleotide-free medium. Leucine zipper domains fused to F1/2 and F3 were cotransfected as positive controls (*23*). TAP1, TAP2, and TAPL were fused to F1/2 or F3 with a 20 amino acid flexible linker between their C-termini and the N-terminus of F1/2 or F3 (Figure 1). The resulting vectors, named TAP1-L_{20}-F1/2, TAP1-L_{20}-F3, TAP2-L_{20}-F1/2, TAP2-L_{20}-F3, TAPL-L_{20}-F1/2, and TAPL-L_{20}-F3, were then paired in different combinations to determine which interactions occur. For example, a TAP1−TAP2 interaction was assayed by cotransfecting TAP1-L_{20}-F1/2 and TAP2-L_{20}-F3 into DHFR-deficient CHO DUKX-B11 cells and selecting for reconstituted DHFR with nucleotide-free medium. Negative controls were leucine zipper DHFR constructs paired with TAP−DHFR contructs, which should not dimerize. Plasmids used in transfections were prepared from TOP10F *Escherichia coli* (Invitrogen) with a QIAfilter plasmid midi kit (Qiagen Inc., Mississauga, Ontario, Canada). CHO DUKX-B11 cells (4×10^5) were plated on 15 cm plates (176 cm^2) and cotransfected with pairs of plasmids using Polyfect (Qiagen) following the manufacturer's instructions. Forty-eight hours later, the medium was exchanged for nucleotide-free medium to select for interacting pairs. Colonies were counted after 10 days of selection.

Western Blots. Crude membranes were prepared by suspending approximately 8×10^5 cells in 500 μL of phosphate-buffered saline (PBS) containing 10 μL of protease inhibitor cocktail (Pharmingen, San Diego, CA) and sonicating for 30 s on ice. Nuclei and whole cells were removed by centrifuging at 500*g* for 5 min at 4 °C. The supernatant was transferred to a new tube, and membranes were pelleted by centrifuging at 48000*g* for 3 h at 4 °C. Pellets were solubilized with 100 μL of 2% sodium dodecyl sulfate (SDS) in PBS without calcium and magnesium (Invitrogen).

The protein concentration was determined via the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Eight micrograms of total protein of each sample was loaded onto a Novex 4−12% Bis-Tris gel (Invitrogen) and run according to the manufacturer's instructions. Anti-DHFR monoclonal antibody was from Research Diagnostics Inc. (Flanders, NJ); the epitope this antibody binds to is proprietary, but our results indicate that it is binding to fragment 3 of DHFR. Anti-TAP1 monoclonal antibody no. 148.3 was a gift from Dr. Robert Tampé (Johann Wolfgang Goethe Universität, Frankfurt am Main, Institute of Biochemistry, Frankfurt, Germany), and anti-TAP2 monoclonal antibody no. 429.5 was a gift from Dr. Peter M. Van Endert (Institut National de la Santé et de la Recherche Médicale, Paris, France). Anti-TAPL polyclonal antibody IB3B was produced in our laboratory. All blots were visualized with either anti-mouse or anti-rabbit HRP-conjugated antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and ECL+ (Amersham Biosciences Corp., Piscataway, NJ).

RNA Purification and Reverse Transcription. Purification of RNA was done with the RNeasy midi kit (Qiagen) following the manufacturer's instructions. To remove any remaining genomic DNA, RNA samples were then treated with deoxyribonuclease I, as per the manufacturer's instructions, except that the concentration of RNA in the reaction was doubled. Reverse transcription (RT) was done using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen) following the manufacturer's instructions using either oligo(dT) (for amplification of full-length hamster DHFR) or the gene-specific primers MUSB2R (5′-TTCCGGTTGTTCAATAAGTC-3′) for murine DHFR fragment F1/2 and MUSD2R (5′-CTTCTCGTAGACTTCAAAC-3′) for murine DHFR fragment F3.

PCR of False Positive Transcripts. cDNAs generated by reverse transcription were used as templates for all PCR reactions. To detect full-length hamster DHFR cDNA, the primer DHUSF (5′-CGCGCCAAACTTGGGGGAAGCACAGCGTAC-3′), complementary to the 5′ untranslated region (UTR), and the primer DHDSR (5′-GGAGGAAAGCAGTAGAACTTGAAGTCAATC-3′), complementary to the 3′ UTR, were used at an annealing temperature of 55 °C. To detect murine DHFR over a hamster DHFR background, murine-specific primers MUSA2F (5′-CGGAGACCTACCCTGGCCTC-3′) and MUSB2R were used at 54 °C for F1/2, and primers MUSC2F (5′-GAATCAACCAGGCCACCTT-3′) and MUSD2R were used at 52 °C for F3. Annealing temperatures, which would specifically amplify murine F1/2 and F3 over endogenous hamster transcript, were determined by temperature gradient PCR (data not shown). Samples were run on Novex 10% TBE gels (Invitrogen) according to the manufacturer's instructions and stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR).

MHC Class I Expression Assay. Cells (4×10^5) were seeded into each well of a 6-well plate (9.6 cm^2). Eighteen hours later, cells were transfected with either TAP1-L_{20}-F1/2 or TAP2-L_{20}-F3 using Polyfect (Qiagen) following the manufacturer's instructions. Cells were grown for 2 days before being trypsinized into single-cell suspensions and spun down at 411*g* into wells of a 96-U-well plate. Cells were then resuspended in 100 μL of medium with 10 μL of R-phycoerythrin-conjugated W6/32 antibody (DakoCytomation, Mississauga, Ontario, Canada) and incubated at 4 °C for 30 min in the dark. Samples were then centrifuged and washed three times with 200 μL of cold PBS (Invitrogen) supplemented with 10% FBS. After resuspension in 500 μL of the wash buffer, the cells were anayzed by fluorescence-activated cell sorting (FACS).

FACS. For detection of DHFR, approximately 8×10^5 cells were incubated in medium containing 10 μM AlexaFluor-488-methotrexate (Molecular Probes) for 17 h. Cells were destained by washing two times with 2 mL of PBS and incubating them in 2 mL of medium without dye for 2 h. Then cells were trypsinized, suspended as single cells, spun down at 500*g*, and resuspended in 1 mL of cold PBS with 10% FBS. Analysis was carried out with 488 nm excitation light and a 525 nm emission filter on a Coulter Epics Elite ESP (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). For detection of MHC class I, stained cells were analyzed with 488 nm excitation light and a 575 nm emission filter on the same machine.

RESULTS

DHFR Protein-Fragment Complementation Assay (PCA) Survival Selection in Nucleotide-Free Medium. Interactions between members of the TAP family were assayed by

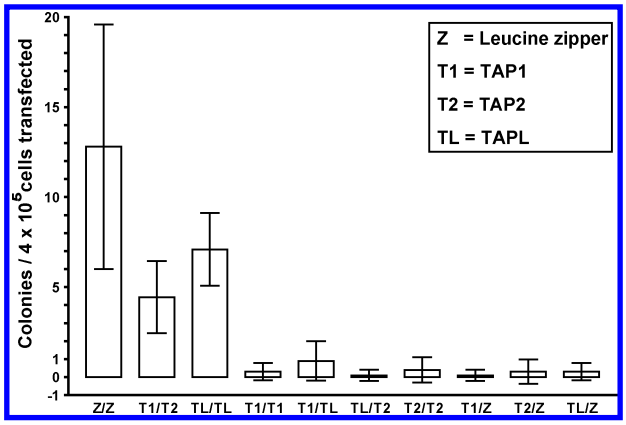

FIGURE 2: Colony counts from survival assay. Pairs of plasmids were cotransfected into DHFR-deficient CHO cells. Forty-eight hours later, the medium was exchanged for nucleotide-free medium to select for interacting pairs. Colonies were counted after 10 days of selection. Bars represent the mean of 10 trials with the standard deviation indicated.

cotransfecting TAP−DHFR constructs with a 20 amino acid flexible linker between the TAP's C-termini and the N-terminus of F1/2 or F3 (Figure 1). The increase of the linker to 20 amino acids was done because early experiments with a 10 amino acid linker did not give significant colony counts above those of negative controls. DHFR constructs with leucine zippers, short proteins that dimerize, were cotransfected as positive controls. Leucine zipper−DHFR constructs paired with TAP−DHFR constructs served as negative controls as they are not expected to interact. As indicated in Figure 2, the standard deviation was quite high between trials of the assay; however, there is a significant and consistent difference between the colony counts of positive and negative controls. Positive control leucine zipper pairs produced 12.8 colonies per 4×10^5 cells. The known TAP1−TAP2 heterodimers yielded an average of 4.4 colonies. TAPL−TAPL produced a mean of 7.1 colonies, suggesting that TAPL forms homodimers. Negative controls (TAPs with leucine zippers) produced between 0.1 and 0.3 colonies. TAP1−TAP1, TAP1−TAPL, TAPL−TAP2, and TAP2−TAP2 produced 0.3, 0.9, 0.1, and 0.4 colonies, respectively. As these values were not significantly higher than those of the negative controls, interactions between these pairs were

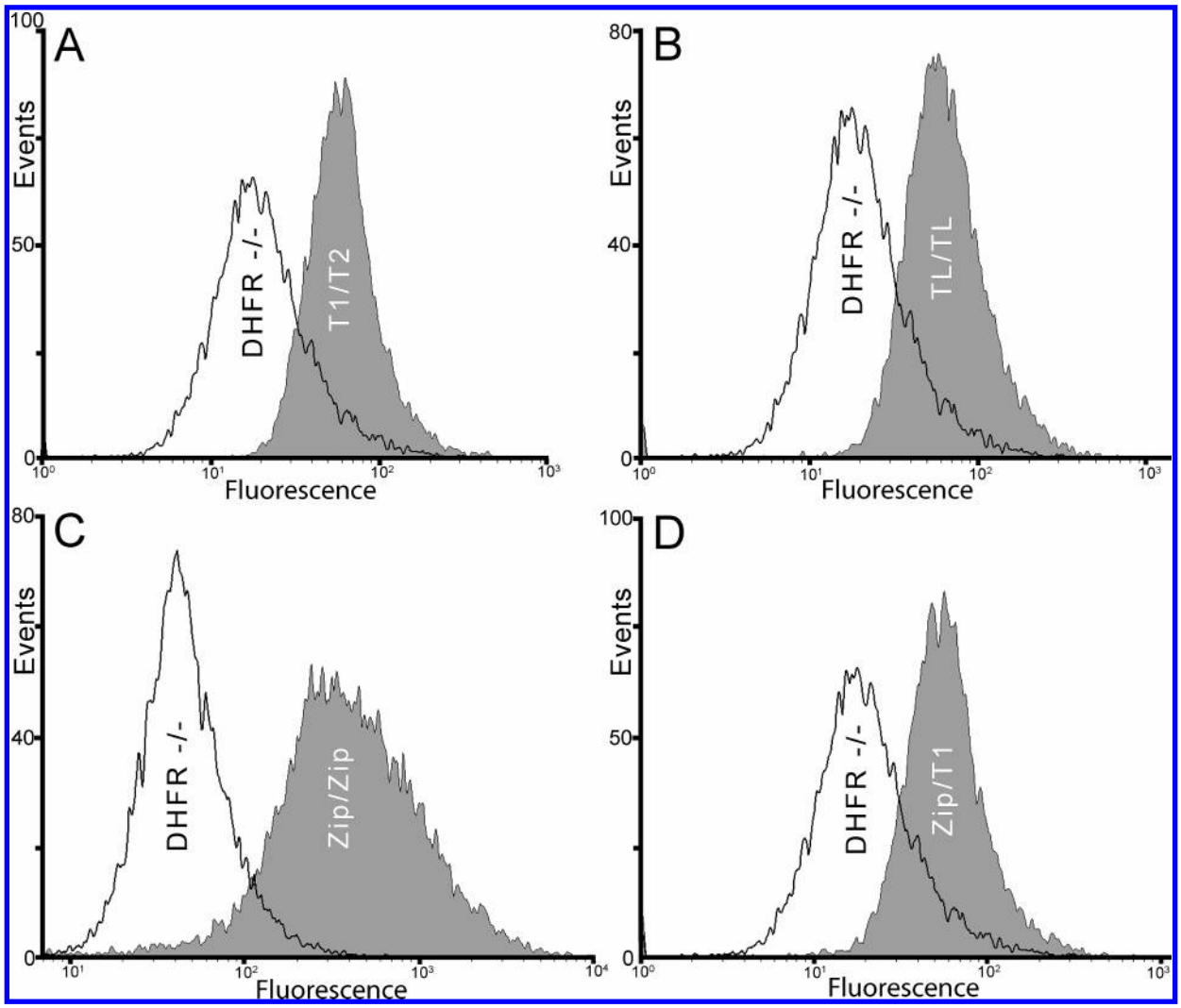

FIGURE 3: Relative increase in fluorescence between nontransfected cells and colonies from survival selection. Histograms labeled DHFR$^{-/-}$ are nontransfected control cells. Filled peaks represent clones of (A) TAP1−TAP2 heterodimers, (B) TAPL−TAPL homodimers, (C) leucine zipper positive controls, and (D) false positive cells transfected with a leucine zipper and TAP1. Cells were incubated overnight (17 h) in AlexaFluor-488-methotrexate. Cells were destained in medium without dye for 2 h. FACS analysis was carried out with 488 nm excitation and 525 nm emission. The zero point for fluorescence is set arbitrarily with each experiment; thus, absolute value comparison cannot be compared between experiments.

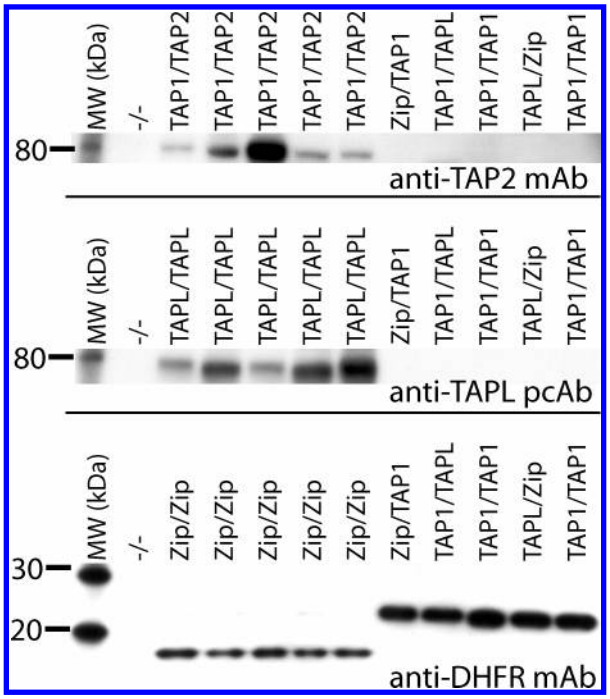

FIGURE 4: Western blot analysis of clones from the DHFR survival assay. Crude lysates were prepared from each clone and run on a 4−12% Bis-Tris SDS−PAGE. Gels were then transferred to PVDF membrane and probed with anti-TAP2 monoclonal, anti-TAPL polyclonal, or anti-DHFR monoclonal antibodies. Lane labels indicate which two DHFR constructs were used in the generation of each clone.

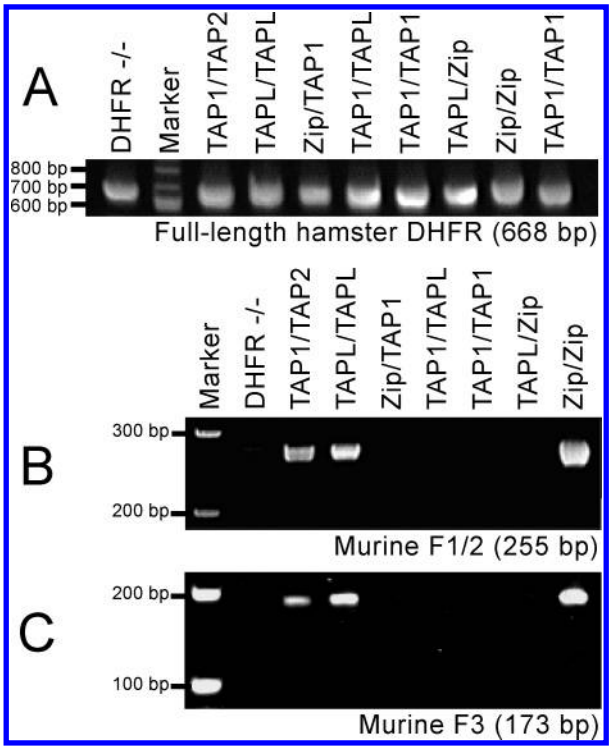

FIGURE 5: PCR detection of transcripts expressed in colonies from the survival assay. (A) Amplification with primers to the 5′ and 3′ UTR of hamster DHFR. (B) Amplification with primers to the F1/2 fragment of murine DHFR. (C) Amplification with primers to the F3 fragment of murine DHFR.

not indicated. Surviving colonies were subsequently cross-validated for the presence of functional DHFR by FACS analysis (see below). The presence of colonies on negative control plates, which indicates that a background of nucleotide-independent false positive colonies can occur, was investigated as well (see below).

FACS Analysis of Clones. As DHFR binds fluorescent methotrexate in a 1 to 1 ratio with high affinity, an increase in fluorescence would confirm the presence of reconstituted DHFR in the cells. Cells from nucleotide-free medium selection assays were stained with AlexaFluor-488-methotrexate and analyzed by FACS with 488 nm excitation light and a 525 nm emission filter. Stained, nontransfected CHO DUKX-B11 cells served as negative controls against which fold increase in fluorescence was calculated. All colonies from the survival assay trapped fluorescent methotrexate, indicating the presence of DHFR. On average, cells were 2.5−5 times more fluorescent than control cells. Four representative FACS histograms, one each for TAP1−TAP2 heterodimers, TAPL−TAPL homodimers, leucine zipper pairs (the positive control), and a false positive colony (leucine zipper with TAP1), are shown in Figure 3.

Western Blot Analysis of Protein Expression in Colonies. To confirm that colony growth was a result of the genes transfected, protein expression from selected clones was analyzed by Western blot (Figure 4). Negative control DHFR-deficient cells, which were not transfected, did not show any bands reactive to TAP1- (not shown), TAP2-, TAPL-, or DHFR-reactive antibodies. When five different TAP1−TAP2 clones were probed with anti-TAP2 antibody, all produced a band of approximately 80 kDa, which is close to the expected size for a 75 kDa TAP2 molecule fused to the 9 kDa F3 fragment of DHFR. When these clones were probed with anti-TAP1 antibodies, a similar band was seen (data not shown). Colonies expected to contain TAPL homodimers also produced a band at approximately 80 kDa, which is also close to the expected size for a 72 kDa TAPL protein fused to the 9 kDa F3 fragment of DHFR. Leucine zipper positive control colonies had the expected 16 kDa band for the zipper-F3 protein. All TAP1−TAP2 and TAPL−TAPL clones were also confirmed by probing with anti-DHFR antibody (data not shown). These results indicate that colonies which grew from the transfection of leucine zipper homodimers, TAP1−TAP2 dimers, and TAPL homodimers do express the appropriate DHFR fusion proteins. However, TAP1−TAP1, TAP1−TAPL, and negative control leucine zipper−TAP colonies all expressed a 21 kDa band, which is the predicted size of full-length DHFR. As full-length DHFR was never transfected into any of these cells, these colonies are considered false positives.

Characterization of Transcripts Expressed in False Positive Colonies. To understand the origin of the false positives, mRNA expression was examined. Using cDNA pools prepared from different cell lines selected in the survival assay, specific primer pairs were used to determine whether mouse-specific or hamster-specific sequences were present in the various clones. Primers specific to the 5′ and 3′ UTRs of hamster DHFR indicated that the full-length transcript for hamster DHFR [expected size = 668 base pairs (bp)] was present in all of the selected clones and in nontransfected CHO DUKX-B11 cells (Figure 5A). The PCR fragments were sequenced and matched the hamster DHFR sequence except for a C → G transversion at position 410 (A of ATG = 1), resulting in an amino acid change of a threonine to an arginine. When these results are coupled with the results from Western analysis, which did not indicate full-length DHFR

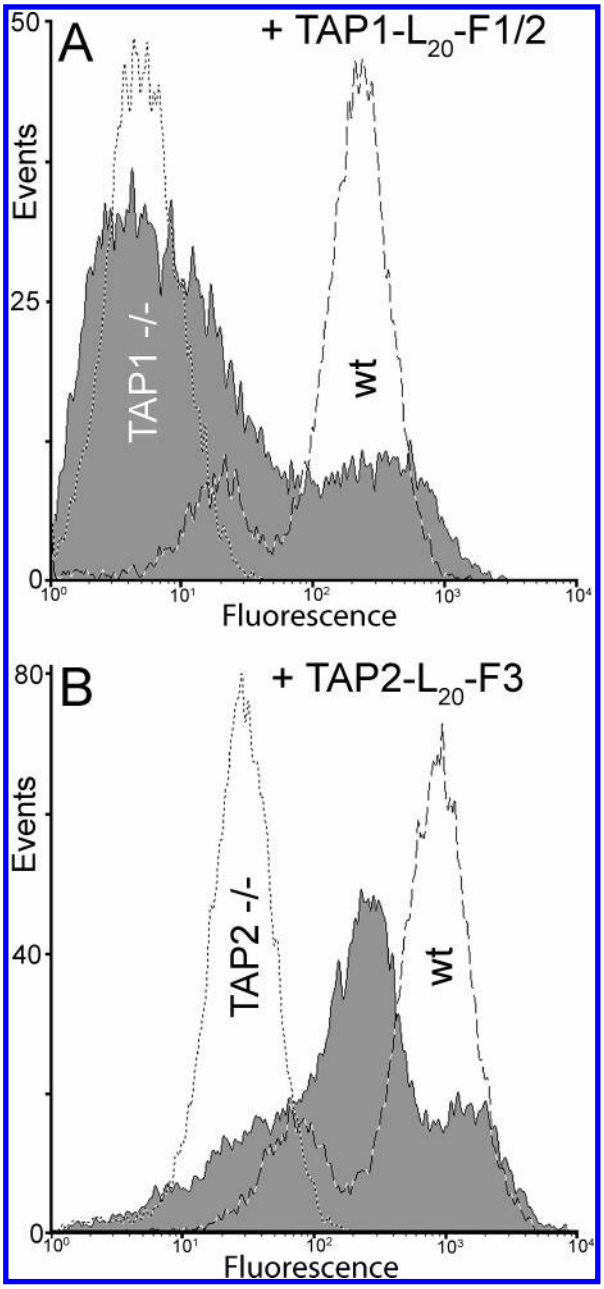

FIGURE 6: FACS of MHC class I expression. The dotted histogram marked TAP1$^{-/-}$ is BRE-169 (TAP1-deficient) cells; the dotted histogram marked TAP2$^{-/-}$ is STF1−169 (TAP2-deficient) cells. Dashed histograms marked wt are STF1−169/TAP1.2 (TAP2-complemented STF1−169) cells, which served as positive controls. The filled peak in (A) is BRE-169 cells transfected with TAP1-L_{20}-F1/2. The filled peak in (B) is STF1−169 cells transfected with TAP2-L_{20}-F3. MHC class I expression was determined by labeling with R-phycoerythrin-conjugated W6/32 antibody. Peaks were generated by FACS analysis with 488 nm excitation and 575 nm emission. The zero point for fluorescence is set arbitrarily with each experiment; thus, absolute value comparison cannot be compared between experiments.

in positive clones or untransfected cells, they indicate that CHO DUKX-B11 cells are DHFR-deficient due to a defect in the translation of the DHFR transcript.

To determine if the false positive colonies arose by re-expressing endogenous hamster DHFR or by recombination of the murine DHFR fragments introduced, we determined which clones contained transcripts with murine DHFR sequences. To detect murine fragments F1/2 and F3, we used murine-specific primers at annealing temperatures which would specifically amplify murine F1/2 and F3 over the endogenous hamster transcript. Using these conditions, we found murine F1/2 fragments (expected size = 225 bp) expressed only in clones from the survival assay which had arisen from the transfection of TAP1−TAP2, TAPL−TAPL, and leucine zipper pairs (Figure 5B). Similarly, murine F3 fragments (expected size = 172 bp) were also expressed only in clones from TAP1−TAP2, TAPL−TAPL, and leucine zipper pairs (Figure 5C). As all the colonies classified as false positives did not express the murine DHFR transcript, the false positive colonies most likely arose from the CHO DUKX-B11 cells reexpressing their endogenous hamster DHFR and not some other genetic event involving the transfected murine DHFR fragments.

Effect of DHFR Fusion on TAP1 and TAP2 Function. Expression of MHC class I on the surface of cells is dependent on peptides being transported by TAP1 and TAP2. When peptide transport is defective, MHC class I heavy chains remain trapped in the ER and, consequently, surface expression of MHC class I is severely reduced. To determine whether TAP1-L_{20}-F1/2 and TAP2-L_{20}-F3 DHFR constructs could function in antigen presentation, we transfected them into TAP1-deficient or TAP2-deficient cell lines to determine if they would facilitate MHC class I expression. Expression of MHC class I was detected by fluorescent W6/32 antibody. Figure 6B shows that the major peak of positive control wild-type-like (STF1−169/TAP1.2) cells is approximately 50 times more fluorescent than the major peak of TAP2-deficient (STF1−169) cells. When TAP2- L_{20}-F3 is transfected into the TAP2-deficient cells, a large increase in fluorescence is observed with a significant proportion of the transfectant population reaching wild-type levels of MHC class I expression. A similar effect is seen when TAP1-L_{20}-F1/2 is transfected into the TAP1-deficient cells (Figure 6A), where we also see a shift in fluorescence with two peaks forming, the second peak resembling that of the STF1−169/TAP1.2 cells. These data suggest that the attachment of fragment F1/2 or fragment F3 to the TAPs does not abolish their ability to function in antigen presentation on MHC class I molecules.

DISCUSSION

This study is the first to directly assay for *in vivo* interactions between all members of the TAP family of human half ABC transporters. We successfully adapted the DHFR PCA to the TAP family and used it to confirm the interaction of TAP1 and TAP2 *in vivo*. While there are much data suggesting that TAP1 and TAP2 form a functional dimer, this is the first time a direct interaction has been shown in intact, living cells. As shown in Figure 2, we found that TAPL forms homodimers; however, we were not able to detect any other interactions between TAP1, TAP2, and TAPL. These results indicate that the transporters in the TAP family form exclusive pairs and that TAP1 does not form homodimers.

False positives were observed in our screens at a rate comparable to those observed for screening of protein−protein interactions by PCA (0.0002%) (*26*). However, the rate of false positives was, in all cases, significantly less than that observed for true positives. Western blot analysis and PCR indicated that these colonies express full-length en-

dogenous hamster DHFR. Because the full-length transcript for hamster DHFR was present in nontransfected CHO DUKX-B11 cells, we can conclude that the DHFR-deficient phenotype of the cells is due to a defect in the translation of the DHFR transcript. How the CHO DUKX-B11 cells were able to re-express a full-length DHFR protein and whether this was promoted by our transfection conditions or treatment of the cells are not known.

Fusing DHFR fragments F1/2 or F3 to the C-termini of TAP genes does not seem to abrogate the function of TAP1 and TAP2. The MHC class I expression assay did not show a complete restoration of a wild-type phenotype when TAP−DHFR fusions were introduced; this is probably due to having less than 100% transfection efficiency and the possibility that TAP−DHFR is less effective than TAP in transporting peptides. Nonetheless, the introduction of TAP1-L_{20}-F1/2 and TAP2-L_{20}-F3 did produce a substantial restoration of MHC class I molecules on the surface of TAP1- and TAP2-deficient cells, suggesting that TAP1− and TAP2−DHFR fusions are both able to transport peptides. These observations are consistent with data indicating that TAP1 with green fluorescent protein at its carboxy terminus is functional (*27*). Thus the DHFR PCA assay has the potential for more detailed functional investigations such as determining if proteins affecting TAP function work by disrupting TAP dimerization. It is also likely that this technology will be fully applicable to the study of other half ABC transporters.

The discovery that TAPL forms homodimers is fundamental to expanding our knowledge about the function of this poorly understood half ABC transporter. It suggests that TAPL can function independently of other half ABC transporters. Thus, we can begin studying TAPL's function, as we know that it homodimerizes to form a "full" ABC transporter. No association between TAPL and TAP1 or TAP2 is in accordance with TAPL residing in lysosomes (*13*), a location where no other mammalian ABC transporters have been found. The recent finding that dendritic cells from TAPL knockout mice have defects in the presentation of heat-inactivated Sendia antigen and ovalbumin peptide on MHC class I molecules (*28*) indicates that TAPL may have a role in the cross-presentation of peptides. Studies to determine if TAPL can transport peptides are currently underway.

Our results fail to show TAP1 forming homodimers, which is in contrast with previous suggestions that TAP1 may function without TAP2 (*15*, *16*). The introduction of rat TAP to small cell lung carcinoma cells (CMT.64 cells) caused them to be recognized by specific CTL when infected with vesicular stomatitis virus (VSV) (*15*). Furthermore, VSV peptides became bound to putative lumenal ER proteins after exogenous rat TAP1 was introduced (*16*). While the data from the CMT.64 cell line are intriguing, they have not been replicated in other cell lines or *in vitro*. Furthermore, CMT.64 cells, which are able to express MHC class I when induced by interferon-γ, do not appear to have any defect in the TAP1 or TAP2 proteins themselves (*29*, *30*). Thus, introduction of surplus TAP1 into these cells may promote the expression or stabilization of TAP2. It may also be that the exogenous TAP1 could contribute to a "leakiness" of the ER membrane in these cells or affect vesicular transport in some way. Thus, while the idea of a TAP1 homodimer was intriguing, it was not supported by our results. It should be noted, however, that if such interactions occur at a very low level (e.g., less than 25 dimers per cell), it would not be detected by the DHFR PCA (*31*).

The discovery that members of the TAP family form exclusive partners raises several questions. Since TAP1 only forms a heterodimer with TAP2, it is interesting that its transcript would be expressed at much higher levels than TAP2 in most tissues (*32*). Also, despite contrary examples such as the white protein in *Drosophila*, where white pairs with either brown or scarlet to transport different substrates (*18*−*21*), we did not find any of the TAP proteins to have more than one dimerization partner. If the transporters do not have multiple dimerization states, then why are half transporters not constructed as full transporters? It could be that it is easier for the organism to regulate expression and function by having half transporters or even that the transport of substrates is regulated by the association and dissociation of half ABC transporters. Another possibility is that encoding the TAP transporter on two separate genes is a way to generate more diversity in a population, and, ultimately, resistance to a wider variety of pathogens.

ACKNOWLEDGMENT

The authors thank the following individuals: Dr. Thomas Spies for providing TAP1 and TAP2 cDNAs; Dr. Henri de la Salle for BRE-169, STF1-169, and STF1-169/TAP1.2 cell lines; Dr. Robert Tampé for anti-TAP1 148.3 antibody; Dr. Peter M. Van Endert for anti-TAP2 429.5 antibody; Dr. Douglas Hogue for assistance with cloning; Denise McDougal for FACS operation; Dr. Tania Kastelic and Dr. Jonathan Sheps for manuscript editing; and Dr. Christoph Bös for helpful discussions.

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BI0491245