## REFERENCES AND NOTES

1. J. E. Parillo, *N. Engl. J. Med.* **328**, 1471 (1993).
2. E. J. Ziegler, *ibid.* **307**, 1225 (1982); R. L. Greenman *et al.*, *J. Am. Med. Assoc.* **266**, 1097 (1991).
3. C. A. Geunter, V. Florica, B. J. Hinshaw, *J. Appl. Physiol.* **26**, 780 (1969); C. R. Raetz, *Annu. Rev. Biochem.* **59**, 124 (1990).
4. B. Beutler and A. Cerami, *Annu. Rev. Biochem.* **57**, 505 (1988).
5. A. H. Ding *et al.*, *J. Immunol.* **141**, 2407 (1988); X. Zang and D. C. Morrison, *J. Exp. Med.* **177**, 511 (1993).
6. S. Okusawa *et al.*, *J. Clin. Invest.* **81**, 1162 (1988); B. Everaedt, P. Broukaet, A. Shaw, W. Fiers, *Biochem. Biophys. Res. Commun.* **163**, 378 (1989).
7. K. Pfeffer *et al.*, *Cell* **73**, 457 (1993); J. Rothe *et al.*, *Nature* **364**, 798 (1993).
8. B. Beutler, I. W. Milsark, A. C. Cerami, *Science* **229**, 869 (1985).
9. K. Ohlsson *et al.*, *Nature* **348**, 550 (1990).
10. S. L. Weinstein, M. R. Gold, A. L. DeFranco, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4148 (1991).
11. K. B. Glaser *et al.*, *Biochem. Pharmacol.* **45**, 711 (1993).
12. Z. Dong, C. A. O'Brien, I. J. Fidler, *J. Leukocyte Biol.* **53**, 53 (1993).
13. M. Kohmo *et al.*, *Biochem. J.* **267**, 91 (1990); A. V. Hoffbrand and R. G. Wickremasinghe, *Blood* **75**, 88 (1990); I. Victor *et al.*, *J. Biol. Chem.* **268**, 18994 (1993).
14. E. Munoz *et al.*, *Eur. J. Immunol.* **22**, 1391 (1992); G. R. Guy *et al.*, *J. Biol. Chem.* **266**, 14343 (1991).
15. P. Yaish, A. Gazit, C. Gilon, A. Levitzki, *Science* **242**, 933 (1988); A. Levitzki, *FASEB J.* **6**, 3275 (1992).
16. T. Iwasaki *et al.*, *FEBS Lett.* **298**, 240 (1992).
17. D. W. Coyne and A. R. Morrison, *Biochem. Biophys. Res. Commun.* **173**, 718 (1990).
18. I. Stefanova *et al.*, *J. Biol. Chem.* **268**, 20725 (1993).
19. R. G. Kilbourn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3629 (1990); R. G. Kilbourn and O. W. Griffith, *J. Natl. Cancer Inst.* **84**, 827 (1992).
20. Z. Dong *et al.*, *J. Immunol.* **151**, 2717 (1993); N. Marczin *et al.*, *Am. J. Physiol.* **265**, H110 (1993).
21. F. Bohmer *et al.*, unpublished results; A. Gazit *et al.*, *J. Med. Chem.* **32**, 2344 (1989); unpublished experiments.
22. S. Tsunawaki and C. F. Nathan, *J. Biol. Chem.* **259**, 4305 (1984). Mice were injected intraperitoneally with 1.0 ml of $NaIO_4$. Macrophages were washed 3 to 4 days later from the peritoneal cavity with phosphate-buffered saline (PBS). After centrifugation at 170*g* for 10 min at 4°C, the cell pellet was resuspended in RPMI 164 containing heat-inactivated newborn calf serum (20%). Adherent macrophage monolayers were obtained by plating of the cells in 96-well microtiter plates at $4 \times 10^5$ cells per well and incubation for 2 hours at 37°C in 5% $CO_2$. Nonadherent cells were removed, and complete medium (RPMI 1640 containing heat-inactivated newborn calf serum) was added. After incubation of macrophage monolayers for 20 hours, tyrphostins were added at a final concentration of 20 μM followed 2 hours later by the addition of *E. coli* 055:B5 LPS (10 μg/ml). Endotoxin (Sigma) was prepared by phenol extraction. After incubation for 6 hours, supernatants were collected and the concentration of TNF-α was determined with an ELISA kit (Endogen). The results are expressed as the mean of two determinators; deviations from the mean did not exceed 8%.
23. Unstimulated macrophages possess basal activity of NO production, which was found to be between 20 and 35% of the maximally LPS-stimulated cells. This activity occurs because the macrophages probably became partially activated during preparation. Tyrphostins suppress this basal activity down to the same level at which they suppress the LPS-induced NO production. The level of basal $NO_2^-$ production seems to depend on the method of macrophage preparation, whereas maximal LPS does not depend on the preparation.
24. D. Wallach *et al.*, *J. Immunol.* **140**, 2994 (1988).
25. The tyrphostins AG 490 and AG 556 were found to be more active in inhibiting TNF-α cytotoxicity in vitro (two- to threefold). This finding suggests that different sets of PTKs mediate the effects of LPS and TNF-α and, therefore, that different families of tyrphostins will be effective against these two agents.
26. S. L. Weinstein *et al.*, *J. Biol. Chem.* **267**, 14955 (1993); Z. Dong *et al.*, *J. Exp. Med.* **177**, 1071 (1993).
27. D. G. Remick *et al.*, *Lab. Invest.* **60**, 766 (1989).
28. M. Gadina *et al.*, *J. Exp. Med.* **173**, 1305 (1991).
29. Inhibition of TNF-α cytotoxicity by tyrphostins. Mouse A9 fibroblasts, a TNF-α–sensitive cell line, were plated in 96-well flat-bottom microtiter plates at 30,000 cells per 0.1 ml to establish a dense monolayer. After incubation for 24 hours at 37°C in a humidified atmosphere with 5% $CO_2$, cycloheximide was added to a final concentration of 50 μg/ml. Cells were treated with murine recombinant TNF-α [$5 \times 10^6$ U/mg (Reportech, Rocky Hill, NJ) at 0.0, 0.2, and 0.5 ng/ml with and without 10 μM tyrphostin. After incubation for 18 hours, the supernatants were aspirated, the monolayers were washed twice with PBS, and 200 μl of neutral red solution (0.02%) was added. After incubation for 2 hours, cells were washed out and the dye that had been absorbed by the live cells was extracted upon the addition of 200 μl of Sorenson buffer containing 50% ethanol. The concentration of the dye was determined by an ELISA autoreader with a 550-nm filter.


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# Interaction of MHC Class I Molecules with the Transporter Associated with Antigen Processing


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**The transporter associated with antigen processing (TAP) delivers cytosolic peptides into the endoplasmic reticulum (ER) where they bind to nascent class I histocompatibility molecules. Class I–peptide complexes are then displayed at the cell surface for recognition by cytotoxic T lymphocytes. Immunoprecipitation of either TAP or class I molecules revealed an association between the transporter and diverse class I products. TAP bound preferentially to heterodimers of the class I heavy chain and $\beta_2$-microglobulin, and the complex subsequently dissociated in parallel with transport of class I molecules from the ER to the Golgi apparatus. The TAP–class I complexes could also be dissociated in vitro by the addition of class I–binding peptides. The association of class I molecules with TAP likely promotes efficient capture of peptides before their exposure to the lumen of the ER.**


Class I molecules of the major histocompatibility complex (MHC) consist of a polymorphic transmembrane heavy chain (~45 kD), a soluble subunit termed $\beta_2$-microglobulin ($\beta_2$M, ~12 kD), and a peptide ligand of eight to ten residues (*1–3*). Peptide ligands are derived predominantly from proteins degraded in the cytosol (*4*), and they must be transported into the lumen of the ER and possibly the cis-Golgi for binding to newly synthesized class I molecules (*5, 6*). Peptide transport is accomplished by the TAP transporter, a heterodimer consisting of the subunits TAP1 and TAP2 (*7, 8*). TAP1 and TAP2 are encoded by genes located in the MHC. Each consists of a transmembrane segment that spans the membrane six to eight times and a proposed cytosolic domain containing an adenosine triphosphate (ATP)–binding cassette (*9*). The TAP1 protein is located on the membranes of the ER and cis-Golgi (*10*). Direct assays of TAP-mediated peptide transport into the ER with the use of either intact microsomes or permeabilized cells reveal that peptides ranging in length from about 8 to 15 residues are transported and some sequence preferences have been noted (*11–13*). Unless bound to a specific class I molecule, peptide ligands for class I are not detectable in cells, which suggests that they may be degraded rapidly in the cytosol and probably also in the ER (*3, 13*). This raises the question of how newly synthesized class I molecules can be efficiently loaded with peptide in the face of other processes competing for peptide, such as rapid degradation, dilution after transport into the ER lumen, and binding to other ER proteins such as the molecular chaperone, BiP (*14*). Presumably, these problems would be minimized if class I molecules and TAP were associated.

Initially, an association between TAP and class I was sought by immunoprecipitating one of these proteins from digitonin-solubilized cells and then testing for the presence of the other protein in the immunoprecipitate by immunoblotting. When lysates from mouse EL4 (*b* haplotype),


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MDAY-D2 (*d* haplotype), and RDM4 (*k* haplotype) cells were treated with TAP2 antiserum (which precipitates TAP2 and associated TAP1), class I H-2K$^b$, K$^d$, and K$^k$ heavy chains, respectively, could be detected in the immunoprecipitates by subsequent immunoblotting with antiserum to H-2K and visualization with $^{125}$I–protein A (~46 kD) (Fig. 1A). The heavy chain of the precipitating immunoglobulin was also detected in varying amounts due to nonspecific binding by $^{125}$I–protein A. Digestion of the TAP2 immunoprecipitates with endoglycosidase H (endo H) revealed that TAP-associated H-2K heavy chains were sensitive to digestion and comigrated with authentic endo H–sensitive heavy chains present in anti–H-2K immunoprecipitates. The endo H–resistant form of K$^d$ could not be resolved from the immunoglobulin (Ig) heavy chain. Thus, at least for K$^b$ and K$^k$, only those class I molecules that have not yet experienced oligosaccharide processing in medial- to trans-regions of the Golgi apparatus are associating with TAP, consistent with the intracellular distribution of the transporter (*10*). That this association is specific and not a result of interactions that occur after cell lysis is supported by the absence of the more abundant endo H–resistant class I molecules in the TAP2 immunoprecipitates.

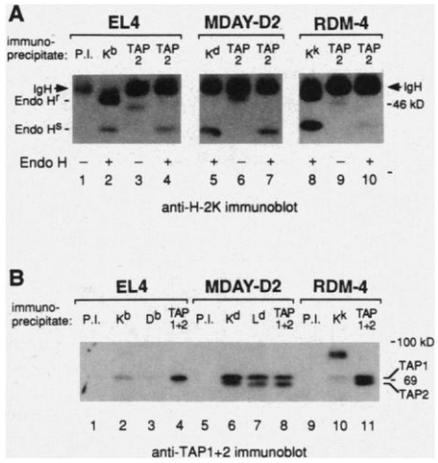


**Fig. 1.** Detection of TAP–class I complexes by co-immunoprecipitation and immunoblotting. (**A**) EL4, MDAY-D2, and RDM-4 cells were lysed in digitonin lysis buffer and class I or TAP2 molecules were immunoprecipitated and digested with endo H as indicated (*34*). Endo H removes immature Asn-linked oligosaccharides from proteins that have not yet been exposed to processing enzymes located in the medial- to trans-Golgi apparatus (*35*). Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), transferred to Immobilon membrane, and immunoblotted with antiserum to peptide 8, which reacts with the COOH-terminal nine amino acids of H-2K heavy chains. The class I immunoprecipitates in lanes 2, 5, and 8 were obtained from one-twelfth, one-sixth, and one-sixth, respectively, of the cell lysate used in the corresponding TAP2 immunoprecipitates. The mobilities of endo H–sensitive (s) and –resistant (r) class I heavy chains are indicated. P.I., preimmune serum. (**B**) Class I or TAP1 and TAP2 molecules were immunoprecipitated from cell lysates as indicated and the TAP proteins in the immunoprecipitates were detected by immunoblotting with antiserum to both TAP1 and TAP2. The TAP1 and TAP2 immunoprecipitates in lanes 4, 8, and 11 were obtained from one-twentieth, one-quarter, and one-twentieth, respectively, of the cell lysate used in the corresponding class I immunoprecipitates.

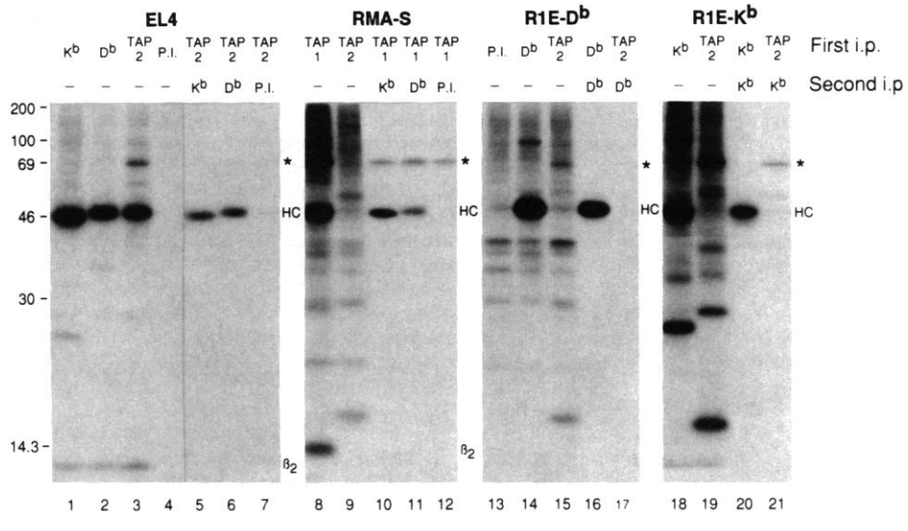


**Fig. 2.** Detection of TAP–class I complexes in cells lacking TAP or class I subunits. Cells were metabolically labeled for 15 min with [$^{35}$S]methionine, lysed in digitonin lysis buffer, and then class I or TAP proteins were immunoprecipitated (First i.p.). In some cases, complexes recovered in the initial immunoprecipitates were disrupted in 0.2% SDS, adjusted to 2% NP-40, and class I proteins were recovered in a second round of immunoprecipitation (Second i.p.) (*36*). The mobilities of TAP (asterisk), class I heavy chain (HC), and $\beta_2$M ($\beta_2$) are shown, as are molecular sizes in kilodaltons. The presence of low amounts of TAP in second immunoprecipitates likely reflects some renaturation of antibodies to TAP in NP-40 (lanes 10 to 12 and 21). A radiolabeled protein of ~25 kD was consistently recovered in K$^b$ immunoprecipitates (lanes 1 and 18). A protein of similar size associates with K$^b$ in a peptide-dependent manner, but it has not yet been identified (*37*). Two proteins of ~27 kD and 15 kD were detected in varying amounts in TAP2 immunoprecipitates (for example, lane 19). They were not specifically associated with TAP, because they were also detected in TAP2 immunoprecipitates of RMA-S cells (lane 9).

In the reciprocal experiment, immunoprecipitates of class I molecules were immunoblotted with antiserum to TAP (Fig. 1B). Proteins reacting with anti-TAP serum and comigrating with authentic TAP1 and TAP2 subunits were detected in association with all class I molecules tested, including H-2K$^b$, -D$^b$, -K$^d$, -L$^d$, and -K$^k$. The TAP subunits from EL4 cells were poorly resolved but could be readily distinguished in the other cell lines. This was a result of differences in the electrophoretic mobility of TAP2, which likely reflects the polymorphisms that have been reported for this subunit (*15*). In the case of RDM4 cells, the H-2K$^k$ molecule was recovered preferentially in association with TAP1 subunit. The additional ~90-kD protein detected in lane 10 appeared to be included nonspecifically in the immunoprecipitate, because it was not detected in replicate experiments.

The nature of the class I–TAP interaction was characterized further by the use of mutant cell lines that lack either TAP or class I subunits and a sensitive, sequential immunoprecipitation technique in which proteins recovered in an initial immunoprecipitate of a metabolically radiolabeled cell lysate were dissociated from the first antibody and then subjected to a second round of immunoprecipitation (Fig. 2). In EL4 cells, which contain normal TAP and class I molecules, immunoprecipitation of TAP1 and TAP2 with antiserum to TAP2 resulted in the isolation of two additional proteins with sizes similar to the class I heavy chain and $\beta_2$M. Solubilization of the TAP immunoprecipitate and re-immunoprecipitation with antibodies to K$^b$ or D$^b$ confirmed that both K$^b$ and D$^b$ were associated with TAP. A similar experiment was performed on RMA-S cells, which lack the TAP2 subunit and accumulate peptide-deficient K$^b$-$\beta_2$M and D$^b$-$\beta_2$M heterodimers intracellularly (*5*, *16*). As expected, no TAP or associated class I proteins could be detected in TAP2 immunoprecipitates (Fig. 2), which con-

firms the specificity of the interaction observed in EL4 cells. However, immunoprecipitation of TAP1 resulted in co-isolation of proteins having the same mobilities as class I heavy chains and $\beta_2$M. Sequential immunoprecipitation confirmed that both $K^b$ and $D^b$ were present in a complex with the TAP1 subunit. Thus, the intact TAP1-TAP2 heterodimer is not required for association with class I molecules.

To determine if TAP interacts with nascent class I molecules before or after the association of heavy chain with $\beta_2$M, free $K^b$ or $D^b$ heavy chains expressed in the $\beta_2$M-deficient R1E cell line (*17*) were tested for their abilities to form complexes with TAP. Although free $D^b$ heavy chains and TAP could be detected in individual immunoprecipitates, no heavy chains were associated with TAP as demonstrated by sequential immunoprecipitation (Fig. 2). The inability to detect TAP-$D^b$ heavy chain complexes was not due to a failure of the antibody to $D^b$ to recognize free $D^b$ heavy chains after solubilization of the TAP2 immunoprecipitate, because the antibody recovered free $D^b$ heavy chains almost quantitatively after solubilization of a $D^b$ immunoprecipitate. The TAP transporter of R1E cells was competent for association with class I molecules because transfection of R1E-$D^b$ (or R1E-$K^b$) cells with the $\beta_2$M gene resulted in the formation of TAP–class I complexes. Consistent with the results for free $D^b$ heavy chains, free $K^b$ heavy chains also did not associate with TAP (Fig. 2). Thus, complexes with TAP appear to be formed only after the nascent class I heavy chain binds $\beta_2$M.

To confirm the stages in class I biogenesis in which interaction with TAP occurs, EL4 cells were radiolabeled biosynthetically for 5 min and TAP–class I complexes were recovered with antibodies to TAP2 from cells that had been lysed at various time points after labeling. For comparison, $\beta_2$M-associated $K^b$ and $D^b$ molecules were isolated from replicate samples with monoclonal antibodies (mAbs) that recognize only $K^b$ or $D^b$ heavy chains associated with $\beta_2$M (Fig. 3A, $K^b$ and $D^b$). Consistent with previous work (*18*), heavy chain–$\beta_2$M association was rapid for both class I molecules; the amount of $\beta_2$M-associated heavy chains was maximal by about 10 min of chase (Fig. 3B, endo $H^s$ $K^b$, $D^b$). Transport from the ER through the medial- and trans-Golgi apparatus had characteristic half-times of ~32 min for $K^b$ and ~62 min for $D^b$, as measured by the processing of heavy chain oligosaccharides to endo H–resistant forms (Fig. 3B insets, endo $H^r$ $K^b$, $D^b$). The association of TAP with $K^b$ and $D^b$ molecules was rapid (Fig. 3A, TAP). Complexes were detected after 5 min of labeling and reached a maximum by 10 min of chase with unlabeled methionine reflecting the association of class I

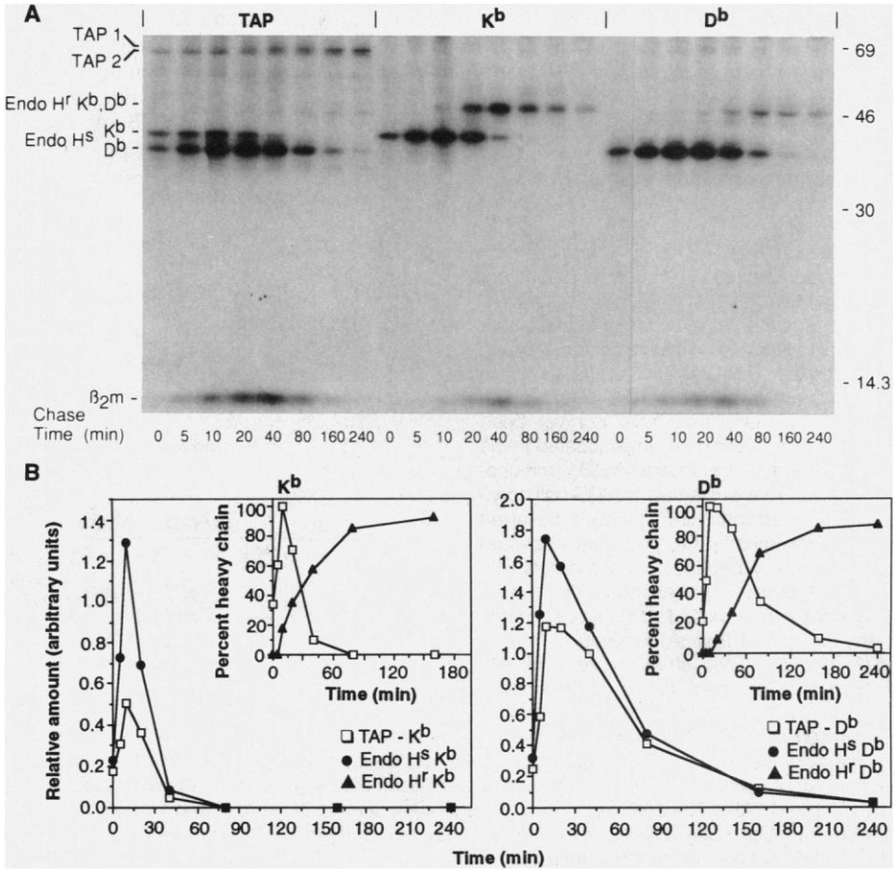


**Fig. 3.** Kinetics of TAP–class I interaction. EL4 cells were radiolabeled for 5 min with [$^{35}$S]methionine and then incubated in methionine-containing medium for periods up to 4 hours. (**A**) At the indicated times, aliquots of cells were lysed and immunoprecipitated with either antiserum to TAP2 to recover TAP–class I complexes, mAb Y-3 to recover $\beta_2$M-associated $K^b$ molecules, or mAb B22-249.R1 to recover $\beta_2$M-associated $D^b$ molecules (*34*). These antibodies recovered in excess of 80% of their respective antigens in a single immunoprecipitation. Immune complexes were digested with endo H and analyzed by SDS-PAGE. The $K^b$ and $D^b$ molecules associated with TAP could be resolved after digestion of TAP2 immunoprecipitates with endo H, because the $K^b$ protein is 10 amino acids larger than $D^b$. The mobilities of TAP, endo H–sensitive and –resistant $K^b$ and $D^b$ heavy chains, and $\beta_2$M are indicated, as are molecular sizes in kilodaltons. (**B**) The amounts of TAP-associated $K^b$ or $D^b$ heavy chains (□) were compared to the amounts of endo H–sensitive $K^b$ or $D^b$ heavy chains (●) by densitometric analysis of the fluorogram in (A). **Insets**: The amounts of TAP-associated $K^b$ or $D^b$ heavy chains (expressed as a percentage of maximum) were compared to the percentage of total $K^b$ or $D^b$ heavy chains at each time point that had acquired resistance to endo H digestion (▲). Because endo H–resistant heavy chains are lost at late time points, due to dissociation of heavy chain and $\beta_2$M (A), the percentage of endo H–resistant heavy chains was determined in a separate experiment using the $\beta_2$M-independent antibodies, anti-peptide 8 ($K^b$), and 28-14-8s ($D^b$).

heavy chains with $\beta_2$M. In agreement with the data in Fig. 1A, only endo H–sensitive $K^b$ and $D^b$ molecules were associated with TAP, indicating that the interaction is restricted to early stages of the secretory pathway. The formation and disappearance of TAP–class I complexes closely reflected the kinetics of formation and disappearance of endo H–sensitive $K^b$ and $D^b$ molecules (Fig. 3B, compare TAP-$K^b$ and -$D^b$ with endo $H^s$ $K^b$, $D^b$). Comparison of the amounts of $K^b$ and $D^b$ heavy chains recovered in the TAP immunoprecipitates with total $\beta_2$M-associated heavy chains at each time point revealed that an average of 52% of $K^b$ and 73% of $D^b$ could be coimmunoprecipitated with TAP. Whether these are true reflections of the percentages of $\beta_2$M-associated class I molecules associated with TAP in vivo or if a portion of the complexes are disrupted upon cell solubilization and antibody binding is unknown. The TAP-$K^b$ and -$D^b$ complexes disappeared at characteristic rates ($t_{1/2}$ ~27 min for $K^b$ and ~67 min for $D^b$) that resembled the ER to Golgi transport rates for the $K^b$ and $D^b$ molecules (Fig. 3B, insets). Thus, dissociation of TAP–class I complexes may be related to events that regulate the ER to Golgi transport of class I molecules.

A possible event that might trigger dissociation of the TAP–class I complex is the binding of peptide ligand to the class I

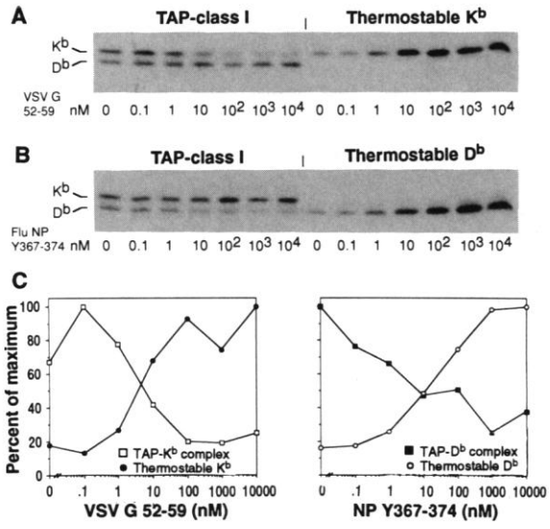


**Fig. 4.** Peptide-induced dissociation of TAP–class I complexes. EL4 cells were radiolabeled for 15 min with [$^{35}$S]methionine and then permeabilized for 7 min in 30 μg/ml digitonin (*38*). The permeabilized cells were resuspended in 0.4 ml of 25 mM Hepes, pH 7.2, 75 mM KOAc, 2.5 mM MgOAc, 1.8 mM $CaCl_2$, 5 mM EGTA, 5 mM ATP and were incubated at 37°C for 20 min with the indicated concentrations of the $K^b$-binding peptide, VSV G 52-59 (**A**) or the $D^b$-binding peptide, Flu NP Y367-374 (**B**) (*39*). Samples were either lysed and immunoprecipitated directly with antibodies to TAP2 to recover TAP–class I complexes (left panels) or they were lysed, heated at 37°C for 30 min, and then immunoprecipitated with mAb Y3 or mAb B22-249.R1 to recover thermostable, peptide-containing $K^b$ or $D^b$ molecules, respectively (right panels). All samples were digested with endo H before analysis by SDS-PAGE. The mobilities of the $K^b$ and $D^b$ heavy chains are indicated. (**C**) The data depicted in (A) and (B) was quantitated by densitometry. The results are representative of two independent experiments.

molecule. The binding of high affinity peptides to heavy chain–$\beta_2$M heterodimers alters the conformation of the complex and enhances its thermal stability (*1, 19*). To test if peptide ligands are required for dissociation of TAP–class I complexes, the plasma membranes of radiolabeled EL4 cells were perforated with a low concentration of digitonin, allowing passage of $K^b$- or $D^b$-specific peptide ligands into the cells during subsequent incubation. As the concentration of peptide increased, the recovery of TAP–class I complexes diminished in corresponding fashion (Fig. 4, A and B, left). The effect was specific to each class I molecule and its peptide ligand, because no loss of TAP-$D^b$ complexes was observed upon incubation with the $K^b$-specific peptide (Fig. 4A) and there was no effect on TAP-$K^b$ complexes of adding the $D^b$-specific peptide (Fig. 4B). Quantitation of the data (Fig. 4C) indicated that 50% of TAP-$K^b$ complexes dissociated at 3.9 nM vesicular stomatitus virus (VSV) G peptide and 50% of TAP-$D^b$ complexes dissociated at 7 nM influenza nucleoprotein (Flu NP) peptide. For both class I molecules, about 20% of the complexes with TAP were refractory to peptide-induced dissociation. Although the specificity of complex dissociation suggested that the loss of complexes was a consequence of peptide binding to class I molecules, further support was obtained by quantitating peptide–class I binding through assessment of the thermostability of $K^b$ and $D^b$ molecules (Fig. 4, A and B, right). At concentrations of peptides similar to those that effected TAP–class I dissociation, $K^b$ and $D^b$ molecules became stable to incubation at 37°C, reflecting the binding of their respective peptide ligands. Half-maximal thermostability was achieved for $K^b$ at 5.2 nM VSV G peptide and for $D^b$ at 10.1 nM Flu NP peptide (Fig. 4C), concentrations similar to the dissociation constants established previously for the binding of these peptides (*20, 21*).

Collectively, our findings suggest a model wherein newly assembled, peptide-deficient heavy chain–$\beta_2$M heterodimers associate with TAP and then dissociate upon binding of a specific peptide ligand to the heavy chain–$\beta_2$M heterodimer. Although peptide binding is clearly necessary to displace TAP from class I molecules, it is not yet clear whether this event alone is sufficient or if other, subsequent events contribute to dissociation of the complex. TAP is capable of transporting peptides as long as 15 amino acids and possibly as long as 33 residues (*11–13, 22*). However, these peptides typically bind with low affinity to class I molecules, suggesting that for some ligands proteolytic trimming in the ER may be required to achieve high affinity binding (*20, 21, 23*). A TAP–class I complex may provide a sufficiently high local concentration of such peptides to permit binding to class I and subsequent trimming of exposed termini (*3*). Although the association between TAP and class I heterodimers is likely to increase the efficiency of peptide capture, peptide binding to class I in vivo can occur in the absence of an interaction with TAP. Peptide ligands fused to hydrophobic signal sequences can be delivered into the ER of TAP-deficient cells and presented in association with class I molecules at the cell surface (*24*). Also, in TAP-deficient cells, class I molecules bind peptides derived from $NH_2$-terminal signal sequences of endogenous proteins of the secretory pathway (*25*).

Our data also bear on the issue of the order of assembly of class I molecules in vivo. Although the bulk of studies supports the association of heavy chains with $\beta_2$M before peptide binding (*20, 26*), the observation that peptides can bind to free heavy chains [albeit with much lower affinity than to heterodimers (*27*)] raises the question of whether this latter pathway occurs significantly in vivo. Because TAP does not interact with newly synthesized free heavy chains but instead associates with heavy chain–$\beta_2$M heterodimers, it seems reasonable to suggest that free heavy chains would be in a less favorable environment to acquire peptides than heterodimers.

Nascent class I heavy chains bind rapidly and quantitatively to calnexin (p88), a resident ER chaperone. This association is maintained during class I assembly and then calnexin dissociates at a rate that is similar to the characteristic ER to Golgi transport rate observed for individual class I allotypes (*18*). Since calnexin retards the intracellular transport of incompletely assembled class I molecules (*28*), it is likely that interaction with calnexin is a significant factor in determining the distinct ER to Golgi transport rates of class I molecules. The relationship between the binding of TAP and calnexin to heavy chain–$\beta_2$M heterodimers has not been established. However, given that TAP is localized to early stages of the secretory pathway (*10*) and it dissociates from class I molecules in parallel with class I transport from the ER to the Golgi apparatus (Fig. 3), the possibility must be considered that TAP may function in conjunction with calnexin in regulating the intracellular transport of assembling class I molecules.

## REFERENCES AND NOTES


1. R. N. Germain and D. H. Margulies, *Annu. Rev. Immunol.* **11**, 403 (1993).
2. V. H. Engelhard, *Curr. Opin. Immunol.* **6**, 13 (1994).
3. H. G. Rammensee, K. Falk, O. Rötzschke, *Annu. Rev. Immunol.* **11**, 213 (1993).
4. L. A. Morrison, A. E. Lukacher, V. L. Braciale, D. P. Fan, T. J. Braciale, *J. Exp. Med.* **163**, 903 (1986); M. W. Moore, F. R. Carbone, M. J. Bevan, *Cell* **54**, 777 (1988); D. F. Hunt *et al.*, *Science* **255**, 1261 (1992); A. L. Goldberg and K. L. Rock, *Nature* **357**, 375 (1992).
5. A. Townsend *et al.*, *Nature* **340**, 443 (1989).
6. J. W. Yewdell and J. R. Bennink, *Science* **244**,1072 (1989); E. J. Baas *et al.*, *J. Exp. Med.* **176**, 147 (1992); C. K. Lapham, I. Bacik, J. W. Yewdell, K. P. Kane, J. R. Bennink, *ibid.* **177**, 1633 (1993); A. Townsend, *Nature* **356**, 386 (1992).
7. A. Kelly *et al.*, *Nature* **355**, 641 (1991); T. Spies *et al.*, *ibid.*, p. 644.
8. T. Spies and R. DeMars, *ibid.* **351**, 323 (1991); S. J. Powis *et al.*, *ibid.* **354, 528 (1991); M. Attaya** *et al.*, *ibid.* **355**, 647 (1992).
9. E. V. Deverson *et al.*, *ibid.* **348**, 738 (1990); J. Trowsdale *et al.*, *ibid.*, p. 741; T. Spies *et al.*, *ibid.*, p. 744; J. J. Monaco, S. Cho, M. Attaya, *Science* **250**, 1723 (1990).
10. M. J. Kleijmeer *et al.*, *Nature* **357**, 342 (1992).

11. J. J. Neefjes, F. Momburg, G. J. Hämmerling, *Science* **261**, 769 (1993); J. C. Shepherd *et al.*, *Cell* **74**, 577 (1993); M. J. Androlewicz, K. S. Anderson, P. Cresswell, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9130 (1993).
12. M.-T. Heemels, T. N. M. Schumacher, K. Wonigeit, H. L. Ploegh, *Science* **262**, 2059 (1993); F. Momburg *et al.*, *Nature* **367**, 648 (1994); T. N. M. Schumacher *et al.*, *J. Exp. Med.* **179**, 533 (1994).
13. F. Momburg, J. J. Neefjes, G. J. Hämmerling, *Curr. Opin. Immunol.* **6**, 32 (1994).
14. F. Lévy, R. Gabathuler, R. Larsson, S. Kvist, *Cell* **67**, 265 (1991).
15. H. R. Gaskins, J. J. Monaco, E. H. Leiter, *Science* **256**, 1826 (1992).
16. Y. Yang *et al.*, *J. Biol. Chem.* **267**, 11669 (1992).
17. D. B. Williams, B. H. Barber, R. A. Flavell, H. Allen, *J. Immunol.* **142**, 2796 (1989).
18. E. Degen and D. B. Williams, *J. Cell Biol.* **112**, 1099 (1991).
19. H.-G. Ljunggren *et al.*, *Nature* **346**, 476 (1990).
20. M. Matsumura, Y. Saito, M. R. Jackson, E. S. Song, P. A. Peterson, *J. Biol. Chem.* **267**, 23589 (1992).
21. E. R. Christinck, M. A. Luscher, B. H. Barber, D. B. Williams, *Nature* **352**, 67 (1991); T. Elliott, J. Elvin, V. Cerundolo, H. Allen, A. Townsend, *Eur. J. Immunol.* **22**, 2085 (1992).
22. R. G. Urban *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1534 (1994).
23. V. Cerundolo *et al.*, *Eur. J. Immunol.* **21**, 2069 (1991).
24. K. Anderson *et al.*, *J. Exp. Med.* **174**, 489 (1991).
25. R. A. Henderson *et al.*, *Science* **255**, 1264 (1992); M. L. Wei and P. Cresswell, *Nature* **356**, 443 (1992).
26. D. B. Burshtyn and B. H. Barber, *J. Immunol.* **151**, 3082 (1993); J. J. Neefjes, G. J. Hämmerling, F. Momburg, *J. Exp. Med.* **178**, 1971 (1993); M.-J. Bijlmakers and H. L. Ploegh, *Curr. Opin. Immunol.* **5**, 21 (1993).
27. T. Elliott, V. Cerundolo, A. Townsend, *Eur. J. Immunol.* **22**, 3121 (1992).
28. M. R. Jackson, M. F. Cohen-Doyle, P. A. Peterson, D. B. Williams, *Science* **263**, 384 (1994).
29. M. H. Smith, J. R. M. Parker, R. S. Hodges, B. H. Barber, *Mol. Immunol.* **23**, 1077 (1986).
30. B. Jones and C. A. Janeway, *Nature* **292**, 547 (1981).
31. K. Ozato and D. H. Sachs, *J. Immunol.* **126**, 317 (1981).
32. H. Lemke, G. J. Hämmerling, U. Hämmerling, *Immunol. Rev.* **47**, 175 (1979).
33. K. Ozato, T. H. Hansen, D. H. Sachs, *J. Immunol.* **125**, 2473 (1980).
34. EL4, MDAY-D2, RDM-4 (18), and transfected R1E cells (17) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. For metabolic radiolabeling, cells were incubated at 37°C in methionine-free RPMI 1640 medium for 30 min and then suspended at $5 \times 10^7$ cells/ml in Met-free medium containing 0.5 mCi/ml [$^{35}$S]Met (>800 Ci/mmol, Amersham). After radiolabeling, aliquots of cells ($3 \times 10^6$ to $1 \times 10^7$) were either lysed directly, or, in the case of pulse-chase experiments, 10 volumes of RPMI 1640 medium containing 1 mM methionine and 10% fetal bovine serum were added and aliquots of $3 \times 10^6$ cells were withdrawn after various periods of time and lysed. For lysis, cells were suspended on ice in 0.5 ml of digitonin lysis buffer (0.5% digitonin, 25 mM Hepes, pH 7.2, 10 mM $CaCl_2$, 10 mM iodoacetamide, 1% aprotinin, and 0.25 mM phenylmethylsulfonylfluoride) for 30 min. Lysates were precleared for 30 min with 1% fixed *Staphylococcus aureus* bacteria and, after centrifugation to remove cell debris and the bacteria, they were subjected to immunoprecipitation for 2 to 16 hours on ice with the following antibodies: For TAP1 and TAP2, rabbit antisera to the COOH-terminal 280 amino acids of TAP1 or the COOH-terminal 273 amino acids of TAP2 (*16*); for free or $\beta_2$M-associated $K^b$, $K^d$, or $K^k$ heavy chains, rabbit antiserum to the peptide encoded by exon 8 of the $K^b$ gene (anti-peptide 8) (*29*); for $\beta_2$M-associated $K^b$ heavy chains, mAb Y-3 (*30*); for free or $\beta_2$M-associated $D^b$ heavy chains, mAb 28-14-8s (*31*); for $\beta_2$M-associated $D^b$ heavy chains, mAb B22-249.R1 (*32*); and for $\beta_2$M-associated $L^d$ heavy chains, mAb 30-5-7s (*33*). Immune complexes were recovered by shaking with protein A–agarose beads for 1 hour and then the beads were washed five times in 0.1% digitonin, 25 mM Hepes, pH 7.2, 10 mM $CaCl_2$. The immune complexes were either eluted directly with SDS-PAGE sample buffer or were first treated with endo H (*28*). Samples were analyzed by SDS-PAGE and visualized by fluorography.
35. A. Velasco *et al.*, *J. Cell Biol.* **122**, 39 (1993).
36. For sequential immunoprecipitation, cells were radiolabeled with [$^{35}$S]Met for 15 min, lysed in digitonin lysis buffer and subjected to a first round of immunoprecipitation (*34*). Immune complexes collected on protein A–agarose were then disrupted by incubating in 0.5 ml of 0.2% SDS in water at 37°C for 1 hour and then samples were adjusted to contain 2% NP-40, 5% skim milk powder, 1 mM tris, pH 7.4, 15 mM NaCl, and 0.1 mM EDTA. Antibody (anti-peptide 8 or 28-14-8s) was then added for a second round of immunoprecipitation as described except that the protein A–agarose beads were washed only once with 1% NP40, 10 mM tris, pH 7.4, 150 mM NaCl, and 1 mM EDTA.
37. V. Cerundolo, T. Elliott, J. Elvin, J. Bastin, A. Townsend, *Eur. J. Immunol.* **22**, 2243 (1992).
38. H. Plutner, H. W. Davidson, J. Saraste, W. E. Balch, *J. Cell Biol.* **119**, 1097 (1992).
39. The Flu NP Y367-374 peptide corresponds to residues 367 to 374 of the influenza nucleoprotein plus a tyrosine at the $NH_2$-terminus (sequence YSNENMETM) and the VSV G 52-59 peptide corresponds to residues 52-59 of the vesicular stomatitis virus G protein (sequence RGYVYQGL) (*2*). Peptides were purchased from the Alberta Peptide Institute and were judged to be more than 95% pure by reversed-phase high-performance liquid chromatography analysis.
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# The TATA-Binding Protein: A General Transcription Factor in Eukaryotes and Archaebacteria

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**The TATA-binding protein TBP appears to be essential for all transcription in eukaryotic cell nuclei, which suggests that its function was established early in evolution. Archaebacteria constitute a kingdom of organisms distinct from eukaryotes and eubacteria. Archaebacterial gene regulatory sequences often map to TATA box–like motifs. Here it is shown that the archaebacterium *Pyrococcus woesei* expresses a protein with structural and functional similarity to eukaryotic TBP molecules. This suggests that TBP's role in transcription was established before the archaebacterial and eukaryotic lineages diverged and that the transcription systems of archaebacteria and eukaryotes are fundamentally homologous.**

The TATA box–binding protein TBP is an essential transcription factor for eukaryotic RNA polymerases I, II, and III, and thus appears to be required for all nuclear transcription (*1*). Binding of TBP to DNA is usually the first step in the assembly of an RNA polymerase II transcription complex. Once bound to DNA, TBP nucleates the assembly of other general transcription factors and directs the start of transcription to a site approximately 25 base pairs (bp) downstream from the TATA box. TBP also functions in transcriptional regulation because it is the target of many transcriptional activators and repressors (*2*, *3*). Reflecting its importance, TBP is exceedingly conserved throughout evolution; its core domain of 180 amino acid residues is over 80% identical between yeast and humans (*4*).

Sequence analysis shows that the three eukaryotic RNA polymerases are evolutionarily related (*5*), which indicates that they arose from a common origin. One might therefore speculate that the ancestor of eukaryotes had a single RNA polymerase and a single set of general transcription factors, one of which would have been TBP. Although the single RNA polymerase of eubacteria is related to the eukaryotic enzymes, eubacteria appear to lack homologs of TBP and of other eukaryotic general factors. The archaebacteria (archaea) constitute a third major kingdom of life (*6*, *7*). Although archaebacteria lack nuclei, resemble eubacteria in morphology, and have a single RNA polymerase, they are at least as distant evolutionarily from *Escherichia coli* as they are from humans. Archaebacterial RNA polymerases more closely resemble those of eukaryotes than those of eubacteria (*7*), and putative archaebacterial homologs of transcription factors TFIIB and TFIIS have been identified (*8*, *9*). Because many archaebacterial gene regulatory sequences map to TATA box–like motifs approximately 25 bp upstream of transcription start sites and fractionation of archaebacterial extracts has identified a transcription factor that operates through

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