

## Cutting Edge: Adenovirus E19 Has Two Mechanisms for Affecting Class I MHC Expression<sup>1</sup>

This information is current as of May 18, 2022.

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*J Immunol* 1999; 162:5049-5052; ;  
<http://www.jimmunol.org/content/162/9/5049>

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## Cutting Edge: Adenovirus E19 Has Two Mechanisms for Affecting Class I MHC Expression<sup>1</sup>

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**Viral strategies for immune evasion include inhibition of various steps in the class I MHC assembly pathway. Here, we demonstrate that adenovirus produces one gene product with a dual function in this regard. It is well established that adenovirus E19 binds class I molecules and retains them in the endoplasmic reticulum (ER). However, E19 also delays the expression of class I alleles to which it cannot tightly bind. Here, we show that E19 binds TAP and acts as a tapasin inhibitor, preventing class I/TAP association.  $\Delta$ E19, an E19 mutant lacking the ER-retention signal, delays maturation of class I molecules, indicating that E19's inhibition of class I/TAP interaction is sufficient to delay class I expression. These data identify tapasin inhibition as a novel mechanism of viral immune evasion and suggest that, through this secondary mechanism, adenovirus can affect Ag presentation by MHC alleles that it can only weakly affect by direct retention. *The Journal of Immunology*, 1999, 162: 5049–5052.**

**V**iruses with mechanisms for evading the immune system have a unique ability to establish long-term infection in the host. The adenovirus E3 19-kDa protein, E19, is one of at least four E3-encoded proteins that attenuate the host immune response to adenovirus infection (1, 2). E19 has been shown to bind class I MHC molecules and prevent their cell surface expression (3, 4), inhibiting the CTL response against adenovirus-infected cells (5). It has been demonstrated that E19 contains a dilysine endoplasmic reticulum (ER)<sup>3</sup>-retention motif in its

cytoplasmic domain (6). The effect of this motif on ER retention of class I molecules has been shown by expression of the deletion mutant  $\Delta$ E19, which lacks the retention motif, in murine mastocytoma cells (7). In the presence of  $\Delta$ E19, a portion of H2-K<sup>d</sup> molecules travel to the cell surface, where they elicit a CTL response. No such response is detected when wild-type E19 is expressed. While this result highlights the ability of E19 to act as an ER-retention molecule, the data also suggest that E19 may be affecting class I expression by an additional mechanism. In the presence of  $\Delta$ E19, the kinetics of K<sup>d</sup> maturation are delayed almost 10-fold, and a significant portion of K<sup>d</sup> molecules fail to reach the cell surface. Additionally, the maturation of those human and murine class I alleles that are bound only weakly by E19 is still greatly delayed in the presence of the viral protein (8, 9). These observations prompted our further investigation into the effect of E19 on the class I assembly pathway.

Class I MHC molecules are assembled in the ER as a membrane-bound heavy chain and a soluble light chain,  $\beta_2$ -microglobulin. Class I heterodimers acquire peptides that are generated in the cytosol by proteasome-mediated degradation and transported into the ER lumen by TAP. Tapasin, an ER-resident membrane protein, facilitates the interaction of class I molecules with TAP by joining the two proteins and cementing the formation of a class I assembly complex, which also includes calreticulin and Erp57. (For a recent review see Lehner and Trowsdale, Ref. 10). We hypothesized that E19 could be inhibiting an additional step in the class I assembly pathway because of the persistent effect of  $\Delta$ E19 on the export of murine class I molecules. To address this question, E19 was expressed in a panel of human cell lines, and the interactions of E19, class I molecules, and TAP were studied. We report here that E19 binds both class I molecules and TAP. Unlike tapasin, however, E19 binds class I molecules and TAP independently rather than simultaneously and, thereby, causes a decrease in class I/TAP association, which can explain the delay in class I maturation we observe in the presence of  $\Delta$ E19.

### Materials and Methods

#### Cell lines, Abs, and virus

EBV-transformed human B lymphoblastoid cell lines (LCL) were cultured in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS at 37°C in 8% CO<sub>2</sub>. The TAP1-/TAP2-negative LCL 721.174 (11), the HLA-A-, -B-, -C-negative LCL 721.221 (12), and the tapasin-negative LCL 721.220 (13, 14) transfected with HLA-B8 were from Dr. Robert DeMars (University of Wisconsin, Madison, WI). HeLa 229 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 2 mM L-glutamine and 10% FBS at 37°C in 6% CO<sub>2</sub>. The specificities of mAb W6/32 (anti-human class I MHC

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Received for publication January 21, 1999. Accepted for publication March 1, 1999.

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<sup>1</sup> This work was supported by National Institutes of Health Grants GM38093 and GM57657 (to F.M.B.), Training Grant GM07175-23 (to E.M.B.), and by a scholarship from Achievement Rewards for College Scientists (ARCS) Foundation (to E.M.B.).

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<sup>3</sup> Abbreviations used in this paper: ER, endoplasmic reticulum; LCL, lymphoblastoid cell line; EndoH, Endoglycosidase H; ECL, enhanced chemiluminescence; NP-Vac, vaccinia virus vector encoding the nucleoprotein of influenza virus; E19-Vac, vaccinia virus vector encoding adenovirus E19;  $\Delta$ E19-Vac, vaccinia virus vector encoding adenovirus E19 with the ER retention signal deleted.

(15)) and Tw1.3 (against adenovirus E19 and  $\Delta$ E19 (7)) and of polyclonal Abs R.RING4C (16) and R.gp48N (14) (against TAP1 and tapasin, respectively, both from Dr. Peter Cresswell, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT), P129-143 (anti-E19, from Dr. William Wold (St. Louis University School of Medicine, St. Louis, MO) (17)), anti-TAP2 (18), and UCSF#2 (anti-class I) (18) have been described. Vaccinia virus constructs encoding the E3 19-kDa protein of adenovirus type 5 (E19-Vac), a deletion mutant of E19 lacking the C-terminal 6 amino acids ( $\Delta$ E19-Vac), and the nucleoprotein of influenza virus (NP-Vac) are described elsewhere (7, 8, 19).

#### Infection and metabolic labeling

HeLa cells ( $1.5 \times 10^7$ ) were infected with vaccinia virus (25 PFU/cell) in 2 ml 0.2% BSA/PBS for 45 min at 37°C. Virus was removed, and cells were maintained in DMEM/10% FBS until 2 h postinfection. LCL ( $5 \times 10^6$ ) were infected with 30 PFU/cell in 1 ml RPMI 1640/1% FBS for 2 h at 37°C. RPMI 1640/10% FBS was added (to  $10^6$  cells/ml), and infection proceeded for 12 h. For  $^{35}\text{S}$  metabolic labeling, cells were starved in cysteine/methionine-free medium supplemented with 5% dialyzed FBS for 1 h at 37°C. Cells were labeled by the addition of 1 mCi per  $10^7$  cells Tran $^{35}\text{S}$ -Label (ICN Pharmaceuticals, Irvine, CA) for 10 min at 37°C, washed three times in ice-cold PBS, and chased in medium containing five times excess methionine at 37°C.

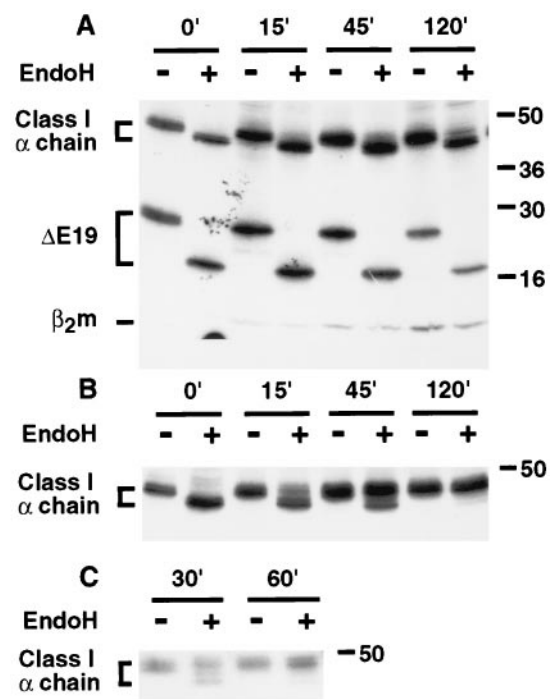
#### Immunoprecipitation and Endoglycosidase H treatment

Cells were lysed in 1% digitonin (Calbiochem, La Jolla, CA), 10 mM Tris (pH 7.4), and 150 mM NaCl on ice for 20 min. After pelleting the nuclei, lysates were precleared with protein G-Sepharose for 1 h at 4°C, then incubated with 2  $\mu\text{g}$  of Ab and fresh protein G-Sepharose for 2 h at 4°C. Immunoprecipitates were washed and analyzed under reducing conditions by SDS-PAGE. For Endoglycosidase H (EndoH) treatment, cells were lysed in 1% Nonidet P-40 (ICN Biomedicals, Aurora, OH) and immunoprecipitated as above. Immunoprecipitates were washed and resuspended in 200  $\mu\text{l}$  100 mM sodium citrate (pH 5.6), 0.2% SDS, and 150 mM 2-ME. Samples were divided in half and incubated at 37°C for 16 h in the presence or absence of 5 mU EndoH (Boehringer Mannheim, Indianapolis, IN) followed by SDS-PAGE analysis. For immunoblotting, proteins were transferred to nitrocellulose, incubated with Ab followed by HRP-conjugated secondary Ab (Zymed Laboratories, South San Francisco, CA), and visualized by enhanced chemiluminescence (ECL) and exposure to film. Bands were scanned and quantitated using the NIH Image 1.61 program.

## Results

### Adenovirus $\Delta$ E19 delays the maturation of human class I MHC molecules

Adenovirus E19 has been shown to bind both human and murine class I MHC molecules (20) and to prevent their cell surface expression (3, 4, 21). Though it lacks an ER-retention motif,  $\Delta$ E19 caused a significant delay in the maturation of murine class I molecules (7). We wanted to determine whether the same delay is observed in human class I expression in the presence of  $\Delta$ E19 and where the delay occurs in the secretory pathway. When  $\Delta$ E19 was expressed in HeLa cells using a vaccinia virus vector ( $\Delta$ E19-Vac), class I molecules remained sensitive to treatment with EndoH throughout a 2-h chase period (Fig. 1A). EndoH cleaves *N*-linked carbohydrates that have not been fully trimmed and terminally glycosylated, modifications which take place during passage through the Golgi complex. Thus, EndoH sensitivity can be used as a measure of a protein's progression from the ER through the secretory pathway. Class I molecules typically begin to acquire EndoH resistance within 30 min (Fig. 1B). Our data show that in the presence of  $\Delta$ E19, human class I molecules, like murine class I molecules, are significantly delayed in export from the ER, remaining EndoH sensitive for longer than 2 h. When cells were infected with a control vaccinia construct encoding the nucleoprotein of influenza virus (NP-Vac), class I molecules acquired EndoH resistance without delay, indicating that vaccinia infection itself does not affect class I maturation (Fig. 1C).

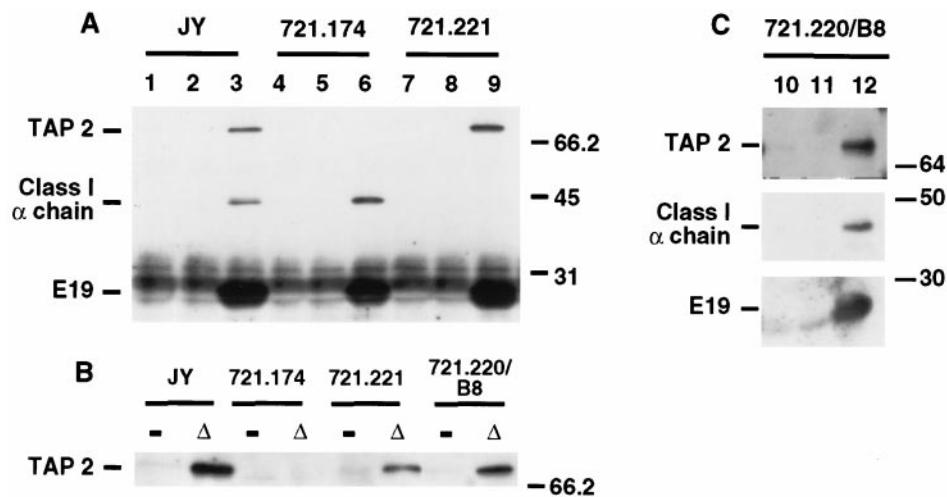


**FIGURE 1.** Delayed maturation of human class I MHC molecules in the presence of adenovirus  $\Delta$ E19. HeLa cells were infected with  $\Delta$ E19-Vac (A) or a control virus (NP-Vac) (C) or not infected (B), then pulse-labeled with [ $^{35}\text{S}$ ]methionine (10 min) and chased for the times indicated (in minutes). Class I MHC molecules were immunoprecipitated from cell lysates with mAb W6/32, which coprecipitates  $\Delta$ E19. Immunoprecipitates were divided in half and either treated with EndoH (+) or left untreated (-), then analyzed by SDS-PAGE and visualized by autoradiography. Migration positions of uncleaved (upper band) and EndoH-cleaved (lower band) class I molecules and  $\Delta$ E19 are indicated at the left, as is the position of  $\beta_2$ -microglobulin. Migration positions of m.w. marker proteins are indicated at the right (in kilodaltons).

### Adenovirus E19 binds TAP

To determine whether the inhibition of class I MHC expression observed in the presence of  $\Delta$ E19 is due to a block in class I assembly, we investigated whether class I molecules bound by E19 associate with TAP. Only properly assembled class I dimers interact with TAP (22). Thus, TAP association can be used to assess whether early steps in the class I assembly pathway are intact. During the course of this investigation, we found an unexpected direct association between E19 and TAP. E19 was expressed in a panel of human B LCL and immunoprecipitated from cell lysates with mAb Tw1.3. Immunoblotting analysis showed that, in the wild-type cell line JY, Tw1.3 coprecipitated both the class I heavy chain and TAP2 with E19 (Fig. 2A). In the class I-negative cell line 721.221 the coprecipitation of TAP with E19 was maintained, indicating that the association of TAP with E19 is not dependent on class I molecules. In the reciprocal experiment, the absence of TAP (721.174 cells) did not prevent the coprecipitation of class I molecules with E19. Identical results were obtained from cells expressing  $\Delta$ E19 (Fig. 2B), showing that E19's ER-retention signal is not required for E19/TAP association. These data suggest that, in addition to binding class I molecules, E19 binds TAP.

While the results from .221 cells indicate that E19 does not associate with TAP indirectly through classical class I MHC molecules, nonclassical class I molecules, known to be expressed in .221 cells (12), could be responsible for the coprecipitation of TAP with E19. Additionally, E19 may bind tapasin and could thereby



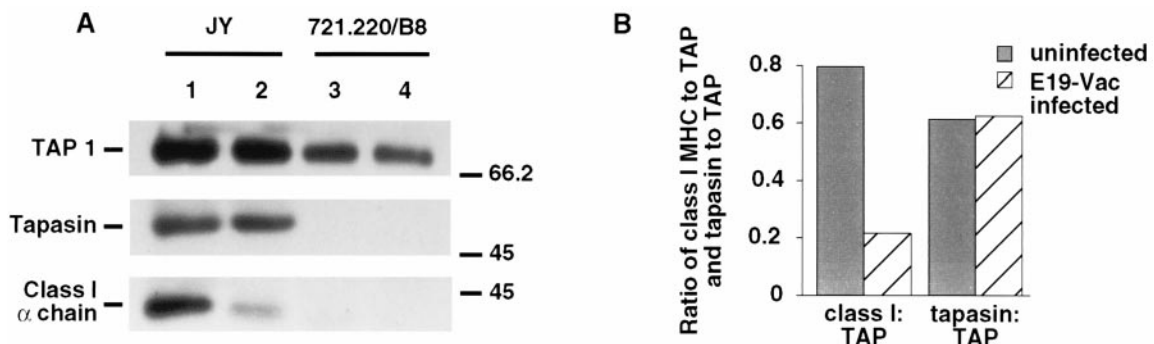
**FIGURE 2.** Adenovirus E19 binds TAP. *A* and *C*, Human LCL, indicated above the lanes, were uninfected or infected with E19-Vac or a control virus (NP-Vac). E19 was immunoprecipitated from cell lysates with mAb Tw1.3. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted for E19 (anti-E19 rabbit serum), class I MHC heavy chain (UCSF#2 rabbit serum), and TAP2 (anti-TAP2 rabbit serum). Migration positions of these proteins are indicated at the left. Proteins were visualized by ECL. Lanes 1, 4, 7, and 10 are uninfected; lanes 2, 5, 8, and 11 are infected with NP-Vac; lanes 3, 6, 9, and 12 are infected with E19-Vac. *B*, LCL were uninfected (–) or infected ( $\Delta$ ) with  $\Delta$ E19-Vac.  $\Delta$ E19 was immunoprecipitated with mAb Tw1.3, and TAP2 was visualized as in *A* and *C*. The migration position of TAP2 is indicated at the left. JY is a wildtype LCL; 721.174 is a TAP-negative LCL; 721.221 is an HLA-A-, -B-, -C-negative LCL; 721.220/B8 is a tapasin-negative LCL that has been transfected with HLA-B8. Migration positions of m.w. markers are indicated at the right of each panel (in kilodaltons).

associate with both classical and nonclassical class I molecules and TAP indirectly. To examine both of these possibilities, we expressed E19 in a tapasin-deficient cell line (721.220/B8). When E19 was immunoprecipitated from .220/B8 lysates, we continued to observe coprecipitation of both class I molecules and TAP (Fig. 2C). In tapasin-deficient .220/B8 cells, this coprecipitation cannot be attributed to tapasin. Nor is it likely to be attributable to E19 interaction with nonclassical class I molecules, since the surface expression of HLA-E has been shown to be tapasin-dependent, which suggests that nonclassical class I molecules are not bound to TAP in these cells (23). Furthermore, the level of expression of nonclassical class I molecules in .221 cells is extremely low compared with total levels of class I molecules in other LCL (12). However, comparable levels of TAP coprecipitated with E19 in .221 and wild-type JY cells (Fig. 2A), strengthening our conclusion

that nonclassical class I molecules are not solely responsible for E19-TAP association. Therefore, we conclude that E19 binds both to class I molecules and TAP.

#### *Adenovirus E19 inhibits the interaction of class I MHC molecules with TAP*

The fact that both class I MHC molecules and TAP coimmunoprecipitated with E19 in tapasin-deficient .220/B8 cells suggested that E19 might be acting as a tapasin mimic. To test this hypothesis, we infected JY and .220/B8 cells with a vaccinia virus vector encoding adenovirus E19 (E19-Vac) and immunoprecipitated TAP from cell lysates with anti-TAP1 rabbit serum. In uninfected JY cells, both tapasin and class I molecules coprecipitated with TAP (Fig. 3A, lane 1). In the absence of tapasin, class I molecules failed to coprecipitate with TAP (Fig. 3A, lane 3). The expression of E19



**FIGURE 3.** Inhibition of class I MHC/TAP interaction by adenovirus E19. *A*, Human LCL, indicated above the lanes, were uninfected (lanes 1 and 3) or infected (lanes 2 and 4) with E19-Vac. Cells were lysed in 1% digitonin, and TAP was immunoprecipitated with anti-TAP1 rabbit serum R.RING4C. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted for TAP1 (R.RING4C) and class I MHC heavy chain (UCSF#2 rabbit serum), and visualized by ECL. The nitrocellulose was then stripped and reprobed for tapasin (R.gp48N rabbit serum). Migration positions of TAP1, tapasin, and class I heavy chain are indicated at the left and of m.w. marker proteins (in kilodaltons) at the right. *B*, Protein bands from uninfected and E19-Vac-infected JY cells in *A* were scanned and quantitated using the National Institutes of Health Image 1.61 program. Ratios of class I heavy chain:TAP and tapasin:TAP were calculated. This quantitation of the data in *A* was reproduced in subsequent experiments and shows a typical decrease in the ratio of class I molecules coprecipitated with TAP in the presence of E19.

in .220/B8 cells did not restore the coprecipitation of class I molecules with TAP, indicating that E19 cannot substitute for tapasin (Fig. 3A, lane 4). This is consistent with lack of sequence homology between the two proteins (24). Interestingly, when E19 was expressed in JY cells, the coprecipitation of class I molecules with TAP was greatly reduced (Fig. 3A, lane 2). Quantitation showed that, in the presence of E19, the ratio of coprecipitated class I molecules to TAP was decreased by ~75%, while the amount of tapasin bound to TAP was approximately the same (Fig. 3B). Taken together, these data indicate that E19 binds to both class I molecules and TAP independently, and in so doing, prevents tapasin from bridging the two molecules. The result is a reduced steady state association between class I molecules and TAP.

## Discussion

These data identify tapasin inhibition as a novel viral strategy for immune evasion and show that tapasin inhibition is a second mechanism by which adenovirus E19 interferes with maturation of class I MHC molecules. We believe E19 evolved this second strategy to inhibit expression of class I alleles to which it cannot tightly bind. Polymorphism of class I molecules poses a considerable challenge to E19 in terms of binding and retaining many different alleles. E19 does, in fact, exhibit allelic specificity toward class I molecules (9, 25). By inhibiting class I/TAP association, E19 can still exert a block on the expression of class I alleles with which it associates only weakly. Like tapasin, E19 is capable of binding both class I molecules and TAP. However, E19 is unable to bridge class I molecules to TAP, and instead appears to bind only one protein at a time. In doing so, however, E19 inhibits the ability of tapasin to join class I molecules to TAP, resulting in a reduced steady state class I/TAP association. This interference with TAP association can explain the delay in class I maturation seen in the presence of  $\Delta$ E19. While tapasin inhibition allows  $\Delta$ E19 to reduce the efficiency of class I assembly, it does not block it completely, as a small portion of class I molecules do become EndoH-resistant over time (Fig. 1A). Thus, E19 provides itself with a backup mechanism enhancing ER retention and inhibition of class I expression.

Inhibition of class I MHC/TAP interaction should lead to a delay in peptide loading of class I molecules. We are unable to detect such a delay, primarily because assays that rely on the thermal instability of empty class I dimers are difficult to interpret. E19 has been shown to bind the  $\alpha 1/\alpha 2$  domains of the class I molecule (9, 25), the same region where peptide binds. E19 and  $\Delta$ E19 remain bound to class I molecules following detergent lysis and heat treatment (data not shown), and, therefore, may be stabilizing empty dimers, rendering them undetectable. However, since we observe both inhibition of class I/TAP association in the presence of E19 and delayed kinetics of class I maturation in the presence of  $\Delta$ E19, the most viable explanation is that E19 delays peptide loading.

Viral immune evasion strategies highlight the evolutionary pressure on viruses to prevent clearance by the host and ensure their continued spread. Blocking Ag presentation is one mechanism by which viruses escape immune detection. In addition to adenovirus, human cytomegalovirus, HSV, and HIV have all been shown to prevent Ag presentation by class I MHC molecules (reviewed by Ploegh, Ref. 26). However, adenovirus E19 is unique in that it is the only viral gene product identified so far that uses more than one mechanism to inhibit class I expression, inhibition of class I/TAP interaction and ER retention. The ability of E19 to inhibit tapasin function appears to be novel, although additional studies will determine whether other viruses use this strategy as well.

## Acknowledgments

We thank Dr. Robert DeMars for supplying B lymphoblastoid cell lines and Drs. William Wold and Peter Cresswell for the gifts of anti-E19, anti-TAP1, and anti-tapasin rabbit sera.

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