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Amyloid Precursor-Like Protein 2 Increases the Endocytosis, Instability, and Turnover of the H2-Kd MHC Class I Molecule

Amit Tuli,*† Mahak Sharma,* Mary M. McIlhaney, † James E. Talmadge, ‡ Naava Naslavsky,* Steve Caplan,* and Joyce C. Solheim2*‡†‡

The defense against the invasion of viruses and tumors relies on the presentation of viral and tumor-derived peptides to CTL by cell surface MHC class I molecules. Previously, we showed that the ubiquitously expressed protein amyloid precursor-like protein 2 (APLP2) associates with the folded form of the MHC class I molecule Kd. In the current study, APLP2 was found to associate with folded Kd molecules following their endocytosis and to increase the amount of endocytosed Kd. In addition, increased expression of APLP2 was shown to decrease Kd surface expression and thermostability. Correspondingly, Kd thermostability and surface expression were increased by down-regulation of APLP2 expression. Overall, these data suggest that APLP2 modulates the stability and endocytosis of Kd molecules. The Journal of Immunology, 2008, 181: 1978–1987.

The presentation of antigenic peptides to T lymphocytes by MHC molecules is essential for recognition and killing of infected and malignant cells. Assembly of MHC class I H chain with antigenic peptide and with the MHC class I L chain, β2-microglobulin (β2m) occurs in the endoplasmic reticulum (ER). Peptide processing and loading of MHC class I molecules involves the participation of several ER proteins: the transporter associated with Ag processing, tapasin, calreticulin, ERp57, Bap 29/31, protein disulfide isomerase, and ER aminopeptidase (1–5). Evidence also indicates that there is regulation of MHC class I trafficking between the ER and the plasma membrane (2, 6–11), although our understanding of this process is relatively limited.

A protein not restricted to the ER that associates with the MHC class I molecule is amyloid precursor-like protein 2 (APLP2) (12–14). APLP2 is a type I transmembrane protein which has a large ectodomain that can be cleaved off and secreted (15). APLP2 is ubiquitously expressed (15), and has a variety of cellular functions, i.e., involvement in mitotic segregation, neurite outgrowth, and epithelial cell migration (16–20). APLP2 is closely related in sequence to amyloid precursor protein (APP), but does not have an β-amloid peptide domain (21–22).

APLP2 was first identified as a protein coimmunoprecipitating with H2-Kd by microsequencing and serological methods (12–13). We have found that transient transfection with APLP2-specific siRNA increases the cell surface expression of Kd, suggesting that normally APLP2 has a down-regulatory effect on Kd surface expression (14). Furthermore, APLP2 interacts with folded Kd molecules and not with open, peptide-free Kd molecules (14), and only associates with Kd in the presence of β2m (13). Notably, APLP2 can be displaced from Kd in cell lysates by the addition of Kd-binding peptides (12), suggesting APLP2 interacts with the α1/α2 domain of Kd.

Our new studies have shown that increased expression of APLP2 reduces the quantity of Kd molecules present at the plasma membrane. We also demonstrated that increased APLP2 expression resulted in greater internalization and more rapid turnover of Kd. In addition, we found that APLP2 binds to the endocytosed Kd molecules. Furthermore, we found that the overall stability of Kd molecules is inversely related to the level of expression of APLP2 in the cell. These data indicate that APLP2 can interact with endocytosed Kd molecules and that it regulates the stability and surface expression of folded Kd molecules.

Materials and Methods

Antibodies

The 34-1-2 mAb recognizes the α1/α2 domain of K3, D3, and D9, and it binds weakly to D0 and L3, but strongly to L4 associated with human β2m and to some L4 mutants with amino acid substitutions in the peptide-binding groove (23–25). The 64-3-7 mAb can detect open, peptide-free L4 (26) and can also detect open forms of other MHC class I H chains, such as K4, into which the 64-3-7 epitope has been introduced. Introduction of the 64-3-7 epitope does not impair peptide presentation, trafficking, or surface expression of K4 or other MHC class I molecules (27–30). The 30-5-7 mAb recognizes L4 molecules with a folded peptide-binding groove (26, 31–34), and the 28-14-8 mAb binds to the α3 domain of L4, D9, D4, and L9 (32, 33, 35). The 34-1-2, 30-5-7, 28-14-8, and 64-3-7 Abs were donated by Dr. T. Hansen (Washington University, St. Louis, MO). The Ab used for APLP2 detection was made against full-length APLP2 (Calbiochem). The Ab recognizing β-actin (PanAb5) was purchased from Novus Biologicals.

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3 Abbreviations used in this paper: β2m, β2-microglobulin; ER, endoplasmic reticulum; APLP2, amyloid precursor-like protein 2; APP, amyloid precursor protein; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Enod, endoglycosidase; EHD1, Eps15 homology domain-containing protein.

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The Journal of Immunology
Cell lines

Cell lines were grown in RPMI 1640 medium (Invitrogen) supplemented with 15% FBS, glutamine, pyruvate, and penicillin/streptomycin. The HeLa cell line was provided to us by Dr. W. Maury (University of Iowa, Iowa City, IA). HeLa cells were transfected with the cDNA encoding Kd with the 64-3-7 epitope (27) in the pIRIS.puro2 vector (BD Biosciences Clontech) or with an Ld cDNA (33) in the RSV.5neo vector (36). As mentioned above, previous studies have established the epitope-tagged Kd molecule as exhibiting normal assembly, folding, and trafficking (27). Selection in medium containing puromycin was performed to generate the stable HeLa-etKd-APLP2, APLP2 and endocytosed Kd were colocalized with the Rab5 dominant negative mutant Rab5Q79L-GFP, which causes early endosomes to be enlarged. HeLa cells stably transfected with Kd and transfected with either Rab5 or Rab5Q79L-GFP were pulsed with anti-Kd Ab 34-1-2 and warmed for 15 min at 37°C. The cells were then incubated with 0.5% acetic acid/500 mM NaCl to strip noninternalized surface-bound 34-1-2 Ab. The cells were fixed with 4% paraformaldehyde, and incubated with rabbit anti-APLP2 serum in staining solution containing saponin, washed, and incubated with fluorescently labeled secondary Abs in the same staining solution. The images were analyzed on a Zeiss LSM 5 Pascal confocal microscope. Red, APLP2; green, Rab5 or Rab5Q79L; blue, Kd, white, colocalized APLP2, Kd, and Rab5 or Rab5Q79L. Bar, 10 μm. Inserts display more highly magnified images of the areas shown in the larger boxes, and the arrows in A indicate vesicles in which APLP2, Rab5, and Kd are colocalized.

Immunoprecipitations and Western blots

Immunoprecipitations and Western blotting were performed by a method similar to a published protocol (39). For protein immunoprecipitations, the cells were washed in PBS containing 20 mM iodoacetamide (Sigma-Aldrich) three times and lysed in 3-[3-cholamidopropyl]dimethylammonio]-l-propanesulfonate (CHAPS) lysis buffer. The CHAPS buffer contained 0.1% CHAPS and 20 mM iodoacetamide and a saturating amount of mAb. After 1 h on ice, the lysates were centrifuged to remove cell nuclei and incubated with protein A-Sepharose beads (GE Healthcare Biosciences). The beads were washed in 0.1% CHAPS/20 mM iodoacetamide in TBS (pH 7.4) four times and boiled in 0.1% CHAPS/20 mM iodoacetamide in TBS (pH 7.4) for 10 min. The eluates were loaded onto SDS-PAGE gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore) for Western blots. After overnight blocking in reconstituted dry milk, membranes were incubated in diluted Ab for 2 h, washed three times with 0.05% Tween 20/PBS, and incubated for 1 h in a dilution of biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag Laboratories). After three 0.05% Tween 20/PBS washes, the membranes were incubated with horseradish peroxidase-labeled swine anti-goat IgG (Zymed) for 1 h, washed with 0.3% Tween 20/PBS three times, and incubated with ECL Western blot developing reagents (GE Healthcare Biosciences). The membranes were exposed to Kodak BioMax film (Eastman Kodak).

When Western blots were performed on cell lysates without an immunoprecipitation step, the cells were washed in PBS containing 20 mM iodoacetamide (Sigma-Aldrich) three times and lysed in buffer containing 0.1% CHAPS (pH 6.8)/2% SDS/12% glycerol/0.02% bromphenol blue to elute the proteins.

The eluted immunoprecipitates were electrohoresed on SDS-PAGE gels (Invitrogen) and transferred to Immobilon-P membranes (Millipore) for Western blots. After overnight blocking in reconstituted dry milk, membranes were incubated in diluted Ab for 2 h, washed three times with 0.05% Tween 20/PBS, and incubated for 1 h in a dilution of biotin-conjugated goat anti-mouse or anti-rabbit IgG (Caltag Laboratories). After three 0.05% Tween 20/PBS washes, the membranes were incubated in diluted streptavidin-conjugated HRP (Zymed) for 1 h, washed with 0.3% Tween 20/PBS three times, and incubated with ECL Western blot developing reagents (GE Healthcare Biosciences). The membranes were exposed to Kodak BioMax film (Eastman Kodak).

When Western blots were performed on cell lysates without an immunoprecipitation step, the cells were washed in PBS containing 20 mM iodoacetamide (Sigma-Aldrich) three times and lysed in buffer containing 0.125M Tris (pH 6.8)/2% (w/v) SDS/12% (v/v) glycerol/0.02% (w/v) bromphenol blue and fresh 0.2 mM PMSF. The lysates were incubated 1 h on ice, then centrifuged to pellet nuclear material. Samples of the supernatants were boiled before loading onto gels. Subsequent steps were performed as described above.

For the endoglycosidase (Endo) H assay, immunoprecipitations were first performed as described above, except proteins were eluted from the
protein A-Sepharose beads by boiling the samples for 5 min in 25 mM Tris (pH 8.3)/0.2 M glycine/0.1% SDS), centrifuging, and transferring the supernatants to fresh tubes. A 10× glycoprotein denaturing buffer (New England Biolabs) was added to 9 μl of supernatant to a final concentration of 1×, and the sample was boiled for 10 min. The sample was then split in half, and the reaction volume of each half was increased by addition of 2 μl of 10 G5 reaction buffer (New England Biolabs), 2 μl of Endo H (New England Biolabs), or 2 μl water, for the mock digestion, and a quantity of

FIGURE 2. Folded Kd molecules internalized from the cell surface could be found colocalized with endogenous APLP2 in vesicles at 10, 20, and 30 min after the start of anti-Kd Ab pulsing. HeLa cells stably transfected with Kd were incubated with anti-Kd Ab 34-1-2 for 0, 10, 20, or 30 min at 37°C. The cells were then treated with 0.5% acetic acid/500 mM NaCl to strip noninternalized surface-bound 34-1-2 Ab. The cells were fixed with 4% paraformaldehyde, and incubated with rabbit anti-APLP2 serum in staining solution containing saponin, washed, and incubated with fluorescein-labeled secondary Abs in staining solution. Images were analyzed on a Zeiss LSM 5 Pascal confocal microscope. Red, APLP2; green, folded Kd; yellow, colocalized APLP2 and endocytosed Kd. Bar, 10 μm. For the 10, 20, and 30 min time points, the insets depict more highly magnified images of the areas shown in the larger boxes, and the arrows in the insets indicate vesicles in which APLP2 and Kd are colocalized.
Increased expression of APLP2 was found to enhance the endocytosis of K4. A, HeLa-etK4 cells (stably expressing K4) were transiently transfected with APLP2-FLAG for 24 h. Anti-K4 Ab 34-1-2 was added and the cells were warmed to 37°C for 20 min. Following Ab internalization, the cells were treated with 0.5% acetic acid/500 mM NaCl to strip off noninternalized surface-bound 34-1-2. The cells were fixed with 4% paraformaldehyde, incubated in staining solution (containing saponin) with rabbit anti-FLAG, washed, and incubated in staining solution and fluorescently labeled secondary Abs, and visualized with a Zeiss LSM 5 Pascal confocal microscope. Red, APLP2; green, folded K4; yellow, colocalized APLP2 and K4. Representative APLP2-transfected cells are outlined with a dashed line. Bar, 10 µm. The insets display more highly magnified images of the areas depicted in the larger boxes. Arrows in the insets point to vesicles in which APLP2-FLAG and K4 are colocalized. B, Results confirming that APLP2 and endocytosed K4 are located together in vesicles were obtained by taking z-section images. Serial z-section images were acquired at 0.4 µm intervals of HeLa-etK4 cells transfected with APLP2-FLAG for 24 h, surface-labeled with 34-1-2, and incubated at 37°C for 15 min. The arrows point to common membrane structures on a representative photomicrograph. APLP2, red; K4, green; merged green and red, yellow. These data confirm that the indicated APLP2- and K4-transfected with APLP2-FLAG for 24 h, surface-labeled with 34-1-2, and incubated at 37°C for 15 min. The arrows point to common membrane structures containing structures are the same endocytic vesicles and not overlaid vesicles. C, APLP2 was bound to endocytosed K4. Lane 1, Lysate of HeLa-etK4 cells plus protein A-Sepharose beads; lanes 2 and 3, 34-1-2 Ab was added to lysates of HeLa and HeLa-etK4 for immunoprecipitation of K4; lane 4, HeLa-etK4 cells transfected with APLP2-FLAG for 24 h were incubated with 34-1-2 Ab for 20 min on ice and then transferred to 37°C for 20 min, noninternalized 34-1-2 Ab was removed by an acid wash, the cells were lysed and centrifuged, and protein A-Sepharose was added to the lysate supernatant; lanes 5 and 6, Isotype control Ab (28-14-8) was added to lysates of HeLa and HeLa-etK4 and an immunoprecipitation procedure was performed; lane 7, HeLa-etK4 cells transfected with APLP2-FLAG for 24 h were incubated with the isotype control Ab 28-14-8 for 20 min on ice and then transferred to 37°C for 20 min, the cells were treated with an acid wash, lysed, and centrifuged, and protein A-Sepharose was added to the lysate supernatant. The samples were electrophoresed on 4–20% acrylamide Tris-glycine gels, transferred to blots, and probed with mAb 64-3-7 that recognizes eK4 (top panel) or with rabbit antiserum against APLP2 (bottom panel). The bands in the 2nd, 5th, and 6th lanes are nonspecific bands. D, Higher expression of APLP2 resulted in increased K4 endocytosis. Image J Software (http://rsb.info.nih.gov) was used to measure the fluorescence of the internalized K4 expressed by >80 cells transfected with APLP2-FLAG and >80 cells not transfected with APLP2-FLAG within the experiment for which confocal data is shown in A. Mean fluorescence intensities and SEs of the mean were calculated, and p values were determined by the use of Student’s paired t test.

Assessment of the K4 turnover rate

For analysis of K4 turnover, a method that was previously described was used (40). Cells were treated with 10 µg/ml cycloheximide and then harvested at 0, 1, 2, 4, or 8 h. Equivalent numbers of live cells were processed as described above for Western blots, and Ab 64-3-7 was used to detect the epitope-tagged K4. Band intensity for K4 was normalized to β-actin band intensity at the same time point. Values were expressed as the percentage of remaining K4 at the 0, 1, 2, 4, or 8 h time point.

Monitoring for induction of stress response

To test whether an increase in expression of APLP2 causes a cellular stress response, we monitored the expression of ER stress proteins, using a published approach (41). For this experiment, we used HeLa-etK4 cells (stably expressing K4) that had been transiently transfected with APLP2-FLAG, transfected with vector only, or were left untransfected with either APLP2 or vector. At 32 h posttransfection, the medium was removed and fresh complete medium was added and the cells were incubated for another 16 h. To generate a positive control, during the 16 h, HeLa-etK4 cells were treated with 2 µg/ml tunicamycin (Sigma-Aldrich). The samples were electrophoresed on 4–20% acrylamide Tris-glycine gels, transferred to blots, and probed with mAb 64-3-7 that recognizes K4 or with rabbit antiserum against APLP2 (top panel) or with rabbit antiserum against APLP2 (bottom panel). The bands in the 2nd, 5th, and 6th lanes are nonspecific bands. D, Higher expression of APLP2 resulted in increased K4 endocytosis. Image J Software (http://rsb.info.nih.gov) was used to measure the fluorescence of the internalized K4 expressed by >80 cells transfected with APLP2-FLAG and >80 cells not transfected with APLP2-FLAG within the experiment for which confocal data is shown in A. Mean fluorescence intensities and SEs of the mean were calculated, and p values were determined by the use of Student’s paired t test.

Biochemical analysis of the binding of APLP2 to endocytosed K4

To demonstrate that APLP2 was bound to endocytosed K4, HeLa-etK4 cells transiently transfected for 24 h with APLP2-FLAG were incubated with 34-1-2 Ab on ice for 20 min and then warmed at 37°C for 20 min. Any 34-1-2 Ab still bound to cell surface K4 was removed by washing with stripping buffer (0.5% acetic acid and 500 mM NaCl), and the cells were lysed. After centrifugation, protein A-Sepharose was added to the lysate supernatant. Several controls were included in the experiment: lysed HeLa-etK4 cells, with no Ab added before or after lysis; HeLa and HeLa-etK4
cells lysed with 34-1-2 or with the 28-14-8 mAb (as an isotype control) in the lysis buffer; and HeLa-etKd cells transiently transfected with APLP2-FLAG and treated with the surface-labeling procedure as described above for HeLa-etKd except that an isotype control Ab (28-14-8) was used instead of 34-1-2. The samples were electrophoresed on 4/3/20% acrylamide Tris-glycine gels and transferred to blotting membranes, which were probed with mAb 64-3-7 to identify etKd or with rabbit antiserum to identify coprecipitated APLP2.

Thermostability assays

The thermostability assay procedure used in this study was designed based on a published procedure (42). For the thermostability assay, cells were washed and lysed with CHAPS buffer and lysates were centrifuged to remove cell nuclei just as described in the section above. Aliquots from the supernatants were incubated at varied temperatures for 12 min on ice or in a Biometra T3 gradient thermocycler (Whatman Biometra). After the thermocycler incubations, immunoprecipitations and Western blots were performed on the aliquots as described in the section above. The amount of Kd in Western blot bands was quantified by densitometry using a Storm (Molecular Dynamics). The relative percentage of folded Kd at each incubation temperature between 25°C and 50°C was calculated after setting the amount of folded Kd at 4°C as 100%.

Flow cytometry assays

In flow cytometry assays, cells were suspended at 5 × 10^6/ml in PBS with 0.2% BSA and 0.1% sodium azide. Cell suspension aliquots in volumes of 0.1 ml were distributed to the wells of a 96-well plate. The cells were incubated with excess mAb or with BSA/azide/PBS alone (as a control) at

FIGURE 4. A, Increased expression of APLP2 did not enhance the endocytosis of folded Ld. The experiment was performed as described in the legend for Fig. 3A, except that HeLa cells stably transfected with Ld were used instead of HeLa-etKd cells, and 30-5-7 (an Ab that recognizes folded Ld molecules) was used instead of 34-1-2. The bar corresponds to 30 μm. B, Quantification of confocal microscopy data demonstrating that higher expression of APLP2 did not increase Ld endocytosis. Image J Software (http://rsb.info.nih.gov) was used to measure the fluorescence of the internalized Ld expressed by >80 cells transfected with APLP2-FLAG and >80 cells not transfected with APLP2-FLAG within the experiment for which confocal data is shown in A. Mean fluorescence intensities and SEs of the mean were calculated, and p values were determined by the use of Student’s paired t test. C, Increased expression of APLP2 did not enhance the endocytosis of open Kd. The experiment was performed as described for Fig. 3A, except that 64-3-7 was used instead of 34-1-2. Bar, 10 μm. D, Increased expression of transferrin receptor did not enhance the endocytosis of folded Kd. The experiment was performed as described for Fig. 3A, except that the cells were transiently transfected with GFP-tagged transferrin receptor (TIR-GFP) instead of APLP2-FLAG. Bar, 10 μm. E, Increased expression of APLP2 does not up-regulate expression of ER stress proteins. HeLa-etKd cells (stably expressing Kd) were transfected with APLP2-FLAG, transfected with vector only, or were left untransfected. APLP2-FLAG-transfected and Vector only transfected cells were not treated with tunicamycin. Tunicamycin (2 μg/ml) was used to induce a stress response in HeLa-etKd cells (lane labeled as “Tun treated”) to create a positive control. The lane corresponding to cells untransfected with APLP2-FLAG or vector and not treated with tunicamycin is labeled as “Untreated.” Western blots of lysates of these cells were probed with an Ab recognizing the KDEL sequence (present on the stress proteins GrP94 and BiP, top panel), an Ab recognizing β-actin (middle panel), or an Ab recognizing APLP2 (bottom panel).
The expression of transfected, tagged APLP2 and the ability of tagged APLP2 to associate with K^d were verified. FLAG-tagged APLP2 [APLP2 (T)] was stably transfected into HeLa-etK^d cells (stably expressing K^d), and the APLP2-FLAG was immunoprecipitated with an anti-FLAG Ab from lysates of these HeLa-etK^d (T) cells. The immunoprecipitated proteins were electrophoresed, transferred to Western blots, and probed with Ab specific for APLP2 (top panel) or with an Ab that recognizes denatured epitope-tagged K^d (64-3-7) (bottom panel). HeLa and HeLa-etK^dpCMV were used as negative controls. B, The population of K^d molecules demonstrated to be bound to APLP2-FLAG included mature (Endo H-resistant) and immature (Endo H-sensitive) forms of K^d. APLP2-FLAG was immunoprecipitated with an anti-FLAG Ab from lysates of HeLa cells stably transfected with etK^d and transiently transfected with APLP2-FLAG (HeLa-etK^d (T) cells). Half of the immunoprecipitate was untreated (−Endo H), and half was incubated with Endo H (+Endo H). Samples from each half were then electrophoresed on 4–20% acrylamide Tris-glycine gels, and Western blots of the electrophoresed samples were probed with an Ab that recognizes denatured epitope-tagged K^d (Ab 64-3-7). C, Stable expression of APLP2 siRNA caused a down-regulation of APLP2 expression and a resultant decrease in the amount of APLP2-associated with stably transfected K^d. A Western blot was probed with an Ab recognizing APLP2, revealing the levels of APLP2 in the indicated cell lysates and bound to K^d immunoprecipitated with 34-1-2. D, Stably increased expression of APLP2 resulted in a decrease in stably transfected cell surface K^d molecules. Thin solid line, HeLa-etK^d plus pCMVTag4A with PE-conjugated secondary Ab only; medium solid line, HeLa-etK^d-APLP2 with secondary-PE; dashed line, HeLa-etK^d-APLP2 with anti-K^d Ab (34-1-2) and secondary-PE; thick solid line, HeLa-etK^d-APLP2 pCMVTagA with 34-1-2 and secondary-PE. E, Stable down-regulation of endogenous APLP2 expression caused an increase in the level of stably transfected K^d molecules at the plasma membrane. Thin solid line: HeLa-etK^d-pSuper1 with secondary-PE; dash-dot-dash line: HeLa-etK^d-APLP2 siRNA with 2 secondary-PE; dotted line, HeLa-etK^d-APLP2 inverse siRNA (i.e., the reversed APLP2 siRNA sequence) with secondary-PE; medium line, HeLa-etK^d-pSuper1 with anti-K^d Ab (34-1-2) and secondary-PE; thick solid line, HeLa-etK^d-APLP2 inverse siRNA with 34-1-2 and secondary-PE; dashed line, HeLa-etK^d-APLP2 siRNA with 34-1-2 and secondary-PE.

Immunofluorescence analysis

To assess APLP2 association with MHC molecules endocytosed from the plasma membrane, cells were grown on glass cover slips, in some cases transiently transfected with APLP2-FLAG using Effectene (Qiagen), and incubated with anti-K^d Ab 34-1-2 at 37°C to allow endocytosis of cell surface K^d and bound Ab. Any Ab still bound to cell surface K^d molecules was then removed by incubation in stripping buffer (0.5% acetic acid/300 mM NaCl) for 90 s and the cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 10 min. Fixed cells were incubated with anti-FLAG or anti-APLP2 rabbit antiserum prepared in staining solution (0.2% saponin/wt/vol/0.5% wt/vol BSA/PBS) for 1 h at room temperature. After 3 PBS washes (5 min/wash), the cells were incubated with a fluorochrome-conjugated mixture of secondary Abs (Alexa Fluor 568 goat anti-rabbit Ab and Alexa Fluor 488 goat anti-mouse Ab) in staining solution for 30 min at room temperature. After three washes in PBS (5 min/wash), the cells were mounted for image analysis. For all immunofluorescence experiments, the images were obtained with a Zeiss LSM 5 Pascal confocal microscope, using a 63× 1.4 numerical aperture lens with appropriate filters.

To ascertain whether the vesicles in which internalized K^d molecules associated with endogenous APLP2 were endosomes, HeLa-K^d cells (stably expressing K^d) were transfected for 24 h with Rab5 or the constitutively active Rab5 mutant Q79L (both GFP-tagged), grown on cover slips, and incubated with anti-K^d Ab 34-1-2 at 37°C for 15 min to allow endocytosis of K^d. After 15 min, any 34-1-2 Ab still bound to cell surface K^d molecules was removed by incubation in stripping buffer for 90 s so that the subsequent immunofluorescence analysis would focus only on the internalized K^d molecules. The cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 10 min and incubated with an anti-APLP2 rabbit antiserum (prepared in saponin-containing staining solution) for 1 h at room temperature. After 3 PBS washes, the cells were incubated with a fluorochrome-conjugated mixture of secondary Abs (Alexa Fluor 568 goat anti-rabbit Ab and Alexa Fluor 488 goat anti-mouse Ab) in staining solution for 30 min at room temperature. After three washes in PBS (5 min/wash), the cells were mounted for image analysis.

Results

Colocalization of APLP2 with folded K^d molecules in endosomal vesicles

Previous studies from our laboratory suggested that APLP2 down-regulated the cell surface expression of K^d (14). To investigate whether the effect of APLP2 on K^d might be mediated through an endocytic mechanism, we analyzed whether APLP2 colocalized with K^d molecules that had been endocytosed from the plasma membrane and with an endosomal marker. An Ab uptake assay was performed with an anti-K^d Ab, using HeLa cells stably transfected with K^d and transiently transfected with Rab5-GFP or Rab5Q79L-GFP. Rab5 is an endosomal protein, and Rab5Q79L is a GTP-locked, nonhydrolyzable Rab5 mutant that stimulates endosomal membrane fusion (38). Expression of Rab5Q79L results...
in the formation of enlarged early endosomes that are accessible to internalized cargo (38), facilitating colocalization analysis. In our assay, we incubated nonpermeabilized cells with anti-Kd Ab 34-1-2 for 15 min at 37°C. After removal of noninternalized 34-1-2 Ab with an acid wash, the cells were permeabilized and stained with Ab against APLP2. Colocalization of endogenous APLP2, internalized Kd, and Rab 5-GFP (Fig. 1A) or Rab5Q79L (Fig. 1B) was noticeable in the endosomes. Thus, APLP2 colocalizes with Kd molecules that have been internalized from the cell surface and that are present within endosomal vesicles.

**APLP2 increased Kd endocytosis**

We also examined the kinetics of the interaction of endogenous APLP2 with Kd molecules endocytosed from the plasma membrane. Anti-Kd mAb 34-1-2 was added to label the cell surface Kd molecules on HeLa-etKd cells (stably expressing Kd), and the cells were incubated for varied amounts of time (0, 10, 20, or 30 min) at 37°C to allow internalization of Kd. The cells were then permeabilized and incubated first with primary Ab against APLP2, washed, and incubated with secondary Abs recognizing the anti-Kd and anti-APLP2 Abs. The 0 min time point is shown as evidence of thorough stripping of noninternalized anti-Kd Ab (Fig. 2). Colocalization of endogenous APLP2 and internalized Kd was apparent by 10 min, and could still be visualized at 20 and 30 min (Fig. 2).

Kd was also colocalized with FLAG-tagged APLP2 (transiently expressed in HeLa-etKd cells) after Kd internalization from the cell surface for 20 min (Fig. 3A). Confocal z-sectioning was done to confirm that internalized Kd and APLP2-FLAG were present in the same endocytic vesicles, and not merely within overlaid ones (Fig. 3B). Furthermore, we demonstrated that APLP2 was bound to endocytosed Kd molecules, as shown by isolation of internalized 34-1-2 Ab and demonstration of APLP2 coimmunoprecipitated with the endocytosed Kd (Fig. 3C). In these experiments, 34-1-2 Ab was incubated with HeLa-etKd cells transiently expressing APLP2-FLAG, the cells were warmed at 37°C for 20 min and then acid stripped and lysed, the samples were electrophoresed, and the 34-1-2-immunoprecipitated Kd and coimmunoprecipitated APLP2 were identified by Western blotting. These data provide biochemical evidence for the binding of endocytosed Kd to APLP2.

Notably, from these confocal studies it could be seen that increased expression of APLP2 resulted in greater endocytosis of Kd. As shown in Fig. 3A, the Kd fluorescence in transiently APLP2-transfected cells (indicated by dashed lines) was greater than in the cells within the same field that were not transfected with APLP2. The graph shown in Fig. 3D displays the mean fluorescence intensity of internalized Kd in cells transiently expressing increased (FLAG-tagged) APLP2 versus cells that only expressed endogenous APLP2 (Fig. 3D). (The data shown in the graph are from the 20-min point in the same Kd uptake assay for which confocal microscopy results are shown in Fig. 3A.) Cells expressing the higher level of APLP2 had significantly more internalized Kd than did cells expressing endogenous levels of APLP2 (Fig. 3D). These findings indicate that APLP2 not only associates with endocytosed Kd, it potentiates its endocytosis. In contrast, elevated APLP2 expression did not significantly increase endocytosis of a different mouse MHc class I molecule, Ld (Figs. 4, A and B). APLP2 binds Ld more weakly than Kd and has significantly less effect on the surface expression of Ld than Kd (data not shown). The effect of APLP2 on Kd endocytosis was specific to the folded form of Kd; there was no increase in open (64-3-7+) etKd internalization when APLP2 was overexpressed (Fig. 4C). In addition, the effect on folded Kd endocytosis was not seen when a different protein (transferrin receptor) instead of APLP2 was overexpressed in HeLa-Kd cells (Fig. 4D).

**Increased expression of APLP2 does not induce expression of stress proteins**

To confirm that expression of an increased amount of APLP2 did not induce a cellular stress reaction, which could conceivably have secondary effects on Kd endocytosis, we examined whether an increase in APLP2 expression led to heightened expression of stress proteins. To assess the stress response, we examined the cellular levels of Grp94 and Bip following APLP2 overexpression. These stress proteins share a carboxy-terminal amino acid sequence (KDEL) that restricts them to the ER and, therefore, they can be detected by an anti-KDEL Ab. HeLa cells stably expressing Kd and untransfected with APLP2, transiently transfected with vector only, or transiently transfected with increased levels of APLP2 were used in these experiments, along with HeLa-etKd cells that had been treated with tunicamycin to induce a stress response (as a positive control). Western blots of lysates of these cell lines were
expression. HeLa-etKd cells (stably expressing Kd) were transiently trans- 
formed with (A) the pCMVTag4A vector (●) or FLAG-tagged APLP2 in 
pCMVTag4A (▲) or (B) APLP2 siRNA (▲) or control siRNA (△). After 
48 h of transfection, the cells were treated with 10 μg/ml cycloheximide for 
0, 1, 2, 4, or 8 h. Equivalent numbers of live cells from each time point 
were lysed and centrifuged, and samples of the supernatants were boiled, 
electrophoresed, transferred to Western blots, and probed with the 64-3-7 
Ab that recognizes denatured epitope-tagged Kd (Ab 64-3-7) or with an Ab 
that recognizes Kd/peptide complexes and augments Kd turnover. To monitor for changes in Kd stability when the APLP2 level was 
increased, lysates of HeLa-etKd cells transfected with APLP2, 
with vector alone, or neither were incubated on ice or at 5°C tem- 
perature intervals between 25°C and 50°C. Following the incubations, folded Kd molecules were immunoprecipitated, visualized by probing the electrophoresed and transferred samples on a West- 
ern blot, and quantified by densitometry. The relative percentage of folded Kd at each incubation temperature (based on setting the amount of folded Kd at 4°C as 100%) was graphed. As shown in 
Fig. 6A, in the presence of an increased level of APLP2 the sta-

tility of folded Kd molecules was reduced. We also performed the 
same type of experiment using lysates of HeLa-etKd cells trans-

FIGURE 7. Kd turnover was augmented as a result of increased APLP2 
expression. HeLa-etKd cells (stably expressing Kd) were transiently trans-
formed with (A) the pCMVTag4A vector (●) or FLAG-tagged APLP2 in 
pCMVTag4A (▲) or (B) APLP2 siRNA (▲) or control siRNA (△). After 
48 h of transfection, the cells were treated with 10 μg/ml cycloheximide for 
0, 1, 2, 4, or 8 h. Equivalent numbers of live cells from each time point 
were lysed and centrifuged, and samples of the supernatants were boiled, 
electrophoresed, transferred to Western blots, and probed with the 64-3-7 
Ab that recognizes denatured epitope-tagged Kd (Ab 64-3-7) or with an Ab 
that recognizes β-actin. The band intensity for Kd was normalized to the 
β-actin band intensity at the corresponding time point. Values are shown 
on the graph for the percentage of remaining Kd at the 0-, 1-, 2-, 4-, and 8-h 
time points.

probed with an Ab against the amino acid sequence KDEL, and 
proteins of the appropriate size to be GrP94 and BiP were detected 
only for the tunicamycin-treated positive control (Fig. 4E). Thus, 
increased expression of APLP2 does not induce expression of the 
stress proteins GrP94 and BiP.

Increased APLP2 expression decreases the quantity of Kd 
molecules on the plasma membrane

Flow cytometric analysis of cells with stably overexpressed, as 
well as stably down-regulated, APLP2 was performed to assess the 
ability of APLP2 to regulate the amount of Kd expressed at the 
cell surface. For these experiments, HeLa-etKd cells stably expressing 
FLAG-tagged APLP2 were generated, and the tagged APLP2’s 
expression and ability to bind Kd were confirmed (Fig. 5A). By 
Endo H assays, we determined that Kd coimmunoprecipitating 
with the transfected APLP2 included both mature (Endo H-resis-
tant) and immature (Endo H-sensitive) forms (Fig. 5B). Stable 
APLP2 siRNA transfectants were also created (Fig. 5C). Overex-
pression of APLP2 following transfection of FLAG-tagged APLP2 
reduced the amount of Kd present at the plasma membrane to 
~60% of the normal level (Fig. 5D). In contrast, HeLa-etKd cells 
that overexpressed the transferrin receptor had Kd expression lev-
els at 99.6% of the normal level (data not shown). The converse 
was also true, in that stable APLP2 siRNA transfectants expressed 
an increased level of Kd at the plasma membrane (~1.7-fold 
higher than the level of Kd on the control cell lines) (Fig. 5E). 
These results indicate that the level of cellular expression of 
APLP2 influences the amount of folded Kd available at the cell 
surface.

Kd thermostability and turnover was affected by the level of 
APLP2

To monitor for changes in Kd stability when the APLP2 level was 
increased, lysates of HeLa-etKd cells transfected with APLP2, 
with vector alone, or neither were incubated on ice or at 5°C tem- 
perature intervals between 25°C and 50°C. Following the incubations, folded Kd molecules were immunoprecipitated, visualized by probing the electrophoresed and transferred samples on a West-
ern blot, and quantified by densitometry. The relative percentage of folded Kd at each incubation temperature (based on setting the amount of folded Kd at 4°C as 100%) was graphed. As shown in 
Fig. 6A, in the presence of an increased level of APLP2 the sta-

Discussion

We have demonstrated that APLP2 associates with MHC class I 
molecules following MHC class I endocytosis. In addition, we 
found that overexpression, as well as reduction, of APLP2 is ca-

pable of modulating MHC class I cell surface expression and sta-
	bility. The finding that cells in which APLP2 is overexpressed 
have decreased cell surface MHC class I expression has implica-

tions for infectious disease and cancer, in that up-regulation of 
APLP2 expression by viruses or tumors could potentially aid in 
escape from CTL surveillance.

Previous studies have provided some insight into the regulation 
of MHC class I molecules beyond the ER. Tapasin has been shown 
to regulate retrograde transport of unstable MHC class I molecules 
back from the Golgi into the ER (11, 43). Spiliotis et al. (8) dem-
strated that the transport of MHC class I molecules from the ER 
to the Golgi is mediated by cargo receptors. It has also been shown 
that when MHC class I molecules are highly overexpressed (20 – 
50-fold), the excess MHC class I molecules could successfully 
traffic as far as the trans-Golgi, but most of the molecules were 
degraded before reaching the plasma membrane (6). Furthermore, 
the cluster of differentiation 99 protein (also known as MIC2) 
seems to be involved in MHC class I transport modulation beyond 
the ER, because it has been found that cluster of differentiation 
99-deficient cells have delayed transport of MHC class I molecules 
between the Golgi and the plasma membrane, resulting in Golgi 
accumulation of MHC molecules (9). All of these findings suggest 
that the level of MHC class I expression at the plasma membrane 
is determined by a series of regulatory steps.

At the plasma membrane, MHC class I molecules have been 
shown to be associated with insulin receptors (44–50). MHC class I 
molecules were found to bring insulin receptors in proximity to 
each other, enhancing their autophosphorylation and phosphoino-
sitide 3-kinase activation (50). Furthermore, when cells were in-
sulin stimulated, MHC class I molecules were also demonstrated to 
be phosphorylated and associated with phosphoinoside 3-kinase 
(50). Because APLP2 can also undergo phosphorylation (51), the 
previous observations with insulin receptor and MHC class I raise
the question of whether the interaction of APLP2 with MHC class I affects the phosphorylation of APLP2 or the MHC class I molecule. APLP2 is known to affect many intracellular pathways through mechanisms which are as yet only poorly understood. Hence, it is conceivable that APLP2 may influence Kβ expression and stability by modulating the action of chaperones in the MHC class I Ag pathway. In this context, possible stress responses that might be induced by APLP2 could be considered. However, our findings do not support any role for APLP2 in inducing stress responses, because our data indicate that increased expression of APLP2 does not support any role for APLP2 in inducing stress responses, beyond the endoplasmic reticulum. J. Mol. Biol. 266: 993–1001.


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