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CD99 Regulates the Transport of MHC Class I Molecules from the Golgi Complex to the Cell Surface[1]

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The down-regulation of surface expression of MHC class I molecules has recently been reported in the CD99-deficient lymphoblastoid B cell line displaying the characteristics of Hodgkin's and Reed-Sternberg phenotype. Here, we demonstrate that the reduction of MHC class I molecules on the cell surface is primarily due to a defect in the transport from the Golgi complex to the plasma membrane. Loss of CD99 did not affect the steady-state expression levels of mRNA and protein of MHC class I molecules. In addition, the assembly of MHC class I molecules and the transport from the endoplasmic reticulum to the *cis*-Golgi occurred normally in the CD99-deficient cells, and no difference was detected between the CD99-deficient and the control cells in the pattern and degree of endocytosis. Instead, the CD99-deficient cells displayed the delayed transport of newly synthesized MHC class I molecules to the plasma membrane, thus causing accumulation of the molecules within the cells. The accumulated MHC class I molecules in the CD99-deficient cells were colocalized with α-mannosidase II and γ-adaptin in the Golgi compartment. These results suggest that CD99 may be associated with the post-Golgi trafficking machinery by regulating the transport to the plasma membrane rather than the endocytosis of surface MHC class I molecules, providing a novel mechanism of MHC class I down-regulation for immune escape. *The Journal of Immunology,* **2001, 166: 787–794.**

Major histocompatibility complex class I protein is expressed on most mammalian cells and presents peptides for T cell immune surveillance. The reduction or lack of MHC class I surface expression can render tumor and virus-infected cells resistant to cytotoxic T cell-mediated killing. Such an immune escape mechanism has been widely demonstrated for malignant tumors (1) and viral infections (2). Recently, several reports have described the acceleration of endocytosis followed by degradation and retention in the *trans*-Golgi network (TGN),[3] which has been exemplified by HIV-1 Nef-dependent down-regulation of surface MHC class I molecules (3). Other possible mechanisms include the defects of transcription, translation, and assembly (4, 5).

The biogenesis of MHC class I complexes is relatively well known (6, 7), owing to the functional studies of their assembly and transport to the cell surface (2, 8, 9). Functional MHC class I complexes contain MHC class I heavy chain, β_2-microglobulin (β_2m) and a peptide and are assembled in the endoplasmic reticulum (ER) and possibly in the *cis*-Golgi. These events are followed by egress from the ER and transport to the proximal Golgi stack of MHC class I complexes (6). Upon leaving the ER, MHC class I molecules have been generally known to rapidly arrive at the cell surface by default pathway without requirements for specific signals (bulk flow) (10). However, recent evidence of sorting of MHC class I molecules in the TGN suggests that the regulated expression of MHC class I molecules at the cell surface can be achieved through the post-Golgi traffic control (11).

CD99 is a ubiquitous 32-kDa transmembrane protein encoded by the *mic2* gene. Although its ligand has not yet been identified, engagement of CD99 with agonistic Ab has been reported to induce the expression of TCR, MHC class I and II molecules on human thymocytes through accelerated mobilization of molecules from the ER or the Golgi compartment to the plasma membrane (12). CD99 is also known to be involved in apoptosis of immature thymocytes (13) and Ewing's sarcoma cell lines (14). In addition, our recent report demonstrated that the down-regulation of CD99 molecules in human B cell lines led to the generation of cells with Hodgkin's and Reed-Sternberg (H-RS) phenotype seen in Hodgkin's disease (HD). The CD99-deficient cell lines displayed the reduction of surface MHC class I molecules, which is one of the typical features of H-RS cells in HD (15). Unlike the cases of tumors and viral infections, little has been known about the mechanism of the down-regulation of MHC class I molecules on the surface of H-RS cells.

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[3] Abbreviations used in this paper: TGN, *trans*-Golgi network; β_2m, β_2-microglobulin; ER, endoplasmic reticulum; H-RS, Hodgkin's and Reed-Sternberg; HD, Hodgkin's disease; Vec-TF, vector transfectants; AS-TF, antisense-CD99 transfectants; Mut, spontaneous CD99-negative mutants; Full-TF, full length-CD99 transfectants; GAM-FITC, FITC-conjugated goat anti-mouse IgG; CaR, calreticulin; endo H, endoglycosidase H.

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Here, we report the regulation of MHC class I molecules by CD99-deficiency, which gains insights on the mechanism of MHC class I down-regulation on the surface of H-RS cells. We found that the loss of CD99 modulated trafficking of MHC class I molecules so that most of the molecules were stagnated in the Golgi compartment. Thus, these observations provide a novel mechanism for the down-regulation of MHC class I expression on the cell surface via the loss of CD99.

Materials and Methods

Cell lines and Abs

Vector transfectants (Vec-TF) and antisense-CD99 transfectants (AS-TF), established by stably transfecting IM-9, an EBV-transformed lymphoblastoid B cell line, with an empty vector or an antisense-CD99 expression construct, respectively, were previously reported (15). A spontaneous CD99-negative mutant IM-9 cell line (Mut) and full length-CD99-transfectants (Full-TF), established by the sorting of spontaneously mutated CD99-negative IM-9 cells and limiting dilution, and by stably transfecting Mut cell line with a full length-CD99 expression construct, respectively, were also used (15). These cell lines were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS. Anti-CD99 mAb, DN16 (DiNonA, Suwon, Korea), was produced in our laboratory previously (12, 15, 16). Anti-human MHC class I mAb, W6/32 hybridoma clone, and FITC-conjugated W6/32 mAb were purchased from American Type Culture Collection (ATCC, Manassas, VA) and Serotec (Oxford, U.K.), respectively. FITC-conjugated goat anti-mouse IgG (GAM-FITC) was obtained from DiNonA (Seoul, Korea). Abs used in the western blotting are as follows: HC10 (anti-human MHC class I heavy chain mAb; gift from Dr. H. L. Ploegh), BBM.1 (anti-β_2m mAb; ATCC), anti-calnexin mAb (clone 37; Transduction Laboratories, Lexington, KY), rabbit anti-human calreticulin (CaR) polyserum (gift from Dr. L. A. Rokeach), and 10C3 (anti-BiP mAb; StressGen, Victoria, Canada)

Flow cytometric analysis

For indirect immunofluorescence staining, cells (5×10^5 per sample) were washed in PBS and incubated with appropriate mAbs for 30 min at 4°C in PBS containing 1% BSA and 0.1% sodium azide. Cells were then washed twice and incubated with GAM-FITC. After staining, cells were fixed in PBS containing 1% paraformaldehyde and analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). For the kinetics of MHC class I surface internalization, the experiments were performed as described elsewhere (16, 17). Briefly, cells were incubated with W6/32 mAb (10 μg/ml in PBS-1% BSA) at 4°C for 60 min, washed, and cultured at 37°C. At different time points, W6/32 mAb-bound MHC class I surface molecules were stained by GAM-FITC, and cells were analyzed by flow cytometry. For the kinetics of MHC class I externalization to the cell surface, cells were incubated with excess amount of W6/32 mAb (250 μg in the 0.5 ml of culture media) for 60 min at 4°C, washed, and transferred to 37°C in culture medium. Cells were then removed at appropriate time points, washed, stained with FITC-conjugated W6/32 mAb (10 μg/ml), and analyzed by flow cytometry.

Northern blot analysis

Total RNA was extracted from Vec-TF and AS-TF cells using TRIzol reagents (Life Technologies, Grand Island, NY). Thirty micrograms of RNA from each sample was electrophoresed, transferred to the membrane, and hybridized with probes labeled by random priming technique. Filters were hybridized, washed under stringent conditions, and developed. HLA-B7, TAP1, TAP2, LMP2 (gifts from Dr. J. Trowsdale), Tapasin (gift from Dr. P. Cresswell), and GAPDH (as an internal control) cDNAs were used as probes.

Western blot analysis

Cells were washed twice in cold PBS and solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min on ice. The lysates were clarified by centrifugation, and then protein concentration was determined using Bradford method (Bio-Rad, Hercules, CA). Total cell lysates (100 μg) were separated by SDS-PAGE (12.5%), transferred to nitrocellulose filter. The filter was probed with HC10, BBM.1, anti-calnexin mAb, rabbit anti-human CaR polyserum, and 10C3. Each reactive protein band was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Pulse-chase, endoglycosidase H (endo H) digestion, and Ab capture assay

Pulse, chase, immunoprecipitation, and endo H digestion were performed as described previously (18), except for minor modifications. Briefly, cells were washed once with PBS, resuspended at 2×10^6/ml in warm methionine- and cysteine-free RPMI 1640 medium containing 10% dialyzed FCS, and incubated at 37°C for 1 h. Cells were then resuspended in warm labeling media containing [^{35}S]methionine/cysteine (Amersham, 7.15 mCi/ml) at 2×10^7/1.0 mCi/ml and labeled for 15 min, followed by incubating the cells in 20-fold excess volume of RPMI 1640 complete medium supplemented with 2 mM each of methionine and cysteine. Samples containing 3×10^6 cells were taken at indicated intervals and washed in cold PBS. Cytoplasmic proteins were extracted using 1% Triton X-100/ TBS (10 mM Tris (pH 7.4), 150 mM NaCl), containing protease inhibitors (1 mM PMSF, 0.1 mM N-α-tosyl-L-lysyl-chloromethylketone (Sigma), 5.0 mM iodoacetamide (Sigma), 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin). The postnuclear supernatant was cleared at 4°C overnight with 5 μl normal mouse serum and 50 μl formalin-fixed *Staphylococcus aureus* (Sigma) and then incubated at 4°C overnight with protein A-Sepharose beads bound with 10 μg/ml W6/32 or HC10 mAbs. After washing in 1% Triton X-100/ TBS buffer three times, samples were subjected to 12.5% SDS-PAGE. Gels were revealed by autoradiography. Endo H digestion experiment was performed according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany). For Ab capture assay, we performed as described previously (17). Briefly, cells were washed three times in PBS and incubated with 10 μg/ml W6/32 mAb on ice for 1 h. Then, Ab-coated cells were washed three times in PBS and extracted using 1% Triton X-100/TBS buffer containing 1 mg/ml BSA and mixed with lysates from unlabeled cells providing 5- to 10-fold excess of MHC class I molecules. Immune complexes were precipitated with protein A-Sepharose beads bound with W6/32 mAb. Two thirds of immunoprecipitates were directly analyzed by SDS-PAGE and autoradiography and the remainder was analyzed by Western blotting with HC10 mAb. For quantification, autoradiographs from three separate experiments were digitally scanned using a Hewlett-Packard flatbed scanner operating (Palo Alto, CA) in transparency mode and the images were analyzed with GS-700 Imaging densitometer. The relative intensities of pixels within experiments were not altered. The background signal was calculated for each lane and subtracted from the ODs of the area corresponding to MHC class I heavy chain bands. The upper band of doublet shown in Fig. 5*B* seems to be a nonspecific band because it also appears in precleared sample (data not shown). Therefore, we excluded the band upon quantification.

Surface biotinylation

To compare the life spans of surface MHC class I molecules between Vec-TF and AS-TF cell lines, we performed the surface biotinylation experiment. Briefly, 5×10^6 cells were chilled in cold PBS and the cell surfaces were biotinyated with a solution containing 1 mg of sulfosuccinimidyl 6-(biotinamido) hexanoate (EZ-Link Sulfo-NHS-LC-Biotin; Pierce, Rockford, IL) in 1 ml of cold biotinylation buffer (20 mM $NaHCO_3$, 150 mM NaCl, pH8.5) at 4°C for 15 min. The reaction was quenched with 50 mM ice-cold glycine in PBS and then washed massively in cold PBS. The cells were resuspended with culture media and returned to the incubator at 37°C. Surface MHC class I molecules were immunoprecipitated with W6/32 mAb using Ab capture assay at indicated time points after the return of the cultures to the incubator. Samples were analyzed by Western blot analysis. Autoradiographs exposed in the linear range of detection were digitally scanned using a Hewlett-Packard flatbed scanner operating in transparency mode and the images were analyzed with GS-700 Imaging densitometer. Data were presented as the percentage of the value at different time points relative to the values obtained at time zero.

Confocal microscopic analysis

Glass coverslip were coated with poly-L-lysine (70–150 kDa; Sigma; 10 μg/ml in distilled water) for 1 h at room temperature, followed by air-dry overnight. Cells (1×10^6/ml) prepared in serum-free media were seeded on glass coverslip and incubated for 30 min at 37°C, and then fixed in 3% paraformaldehyde-PBS for 20 min at room temperature and incubated for 10 min in 50 mM NH_4Cl to quench-free aldehydes. After permeabilization in 0.1% Triton X-100-PBS for 15 min, cells were incubated for 30 min in blocking solution (10% human serum in PBS) followed by incubation with appropriate Abs in blocking solution. Steady-state MHC class I levels were visualized by incubation with FITC-conjugated W6/32 mAb, recognizing assembled human MHC class I molecules. In localization experiments, cells were first incubated with anti-γ-adaptin mAb (Transduction Laboratories), or with a rabbit polyserum, which reacts with α-mannosidase II in

Golgi complex, followed by incubation with either PE-conjugated goat anti-mouse IgG for anti-γ-adaptin mAb or anti-rabbit IgG for anti-mannosidase II polyserum. Subsequently, cells were blocked with 5% normal mouse serum and stained with FITC-conjugated W6/32 mAb. The stained cells were examined by immunofluorescence confocal microscopy (Bio-Rad 1024; Bio-Rad).

Results

Lack of CD99 down-regulates surface MHC class I expression

Previously, we described a stable CD99-deficient cell line (AS-TF) that was produced by transfection of an antisense-CD99 expression construct into IM-9 (15). The AS-TF cell line is characterized by a complete loss of mRNA and protein of CD99, subsequently, resulting in the absence of CD99 on the cell surfaces (15) (Fig. 1*A*; CD99). The AS-TF cells displayed markedly reduced expression of MHC class I molecules on the cell surface in comparison with the Vec-TF cells (Fig. 1*A*). Accordingly, the surface expression of β_2m, the second polypeptide component of MHC class I complex, was also reduced to the similar extent in the CD99-deficient cell lines (data not shown). In contrast, the expression levels of other surface molecules, such as ICAM-1 (Fig. 1*A*), CD46 (data not shown), and CD45RA (15), on AS-TF cells, remained unaltered. Because the data imply that the loss of CD99 specifically induces the decrease in surface MHC class I proteins, we examined whether two events are directly linked so that the decreased MHC class I expression could be restored by forced expression of CD99. We performed the FACS analysis of a spontaneous CD99-negative mutant IM9 cell line (Mut) in comparison with the cell line in which CD99 expression was restored by transfecting the CD99-expression plasmid (Full-TF). The result of the analysis with Full-TF cells revealed that when CD99 reappeared on the cell surface, the MHC class I expression also became restored (Fig. 1*B*). It is intriguing that the surface level of CD99 expression has a close relation with that of MHC class I expression, indicating that CD99 may directly influence on the level of cell surface expression of MHC class I molecules.

CD99 deficiency does not affect steady-state levels of mRNAs and proteins of MHC class I subunits or of MHC class I assembly-related molecules

Because it was observed that CD99 deficiency caused the decrease in the level of surface MHC class I expression, we then investigated the possibilities of any defects in molecules related to the surface expression of class I molecules that might be influenced by CD99. First, RNA was extracted from AS-TF and Vec-TF cell lines to compare the amounts of MHC class I heavy chain transcripts. There was no difference between AS-TF and Vec-TF cells at the levels of mRNA expression (Fig. 2*A*). Moreover, steady-state mRNA levels of TAP1, TAP2, LMP2, and Tapasin (19, 20) of AS-TF cells were not lower than those of Vec-TF cells (Fig. 2*A*), although TAP, LMP, a subset of the proteasome β subunits, and Tapasin molecules facilitating MHC class I assembly have been known to affect the level of cell surface MHC class I molecules (21–24). Upon quantification, even higher levels of TAP1, TAP2, and LMP2 mRNA were observed in AS-TF cells, but the reason or effect of the up-regulation in AS-TF cells is currently uncertain. Next, we performed Western blot analyses to examine whether there is the reduction of MHC class I subunits and chaperones, such as calnexin, CaR, and Bip, in the translational level in the AS-TF cells. When the total protein of equal amount was loaded, the analyzed proteins showed similar or slightly increased protein levels rather than decreased protein levels in AS-TF cells in comparison with Vec-TF cells (the ratios of Vec vs AS were in the range of 1:1.05–1.3, as analyzed by densitometer). Among them, in the case of β_2m, more than 3-fold increase at the protein level was seen in AS-TF cells (Fig. 2*B*). The reason of the marked increase in the expression of β_2m in AS-TF cells remains to be identified. These results clearly indicate that the down-regulation of MHC class I molecules on the surface of AS-TF cells is not due to any quantitative decrease of MHC class I subunits nor MHC class I assembly-related proteins.

CD99 deficiency does not affect the transport through the ER to the cis/medial-*Golgi compartment*

Down-regulation of surface MHC class I molecules has been explored by many viral proteins through interference with not only the assembly of functional class I complex in the ER but also transport to the Golgi complex (2, 8). Therefore, we examined the possibility of down-regulating the transport rate from the ER to the

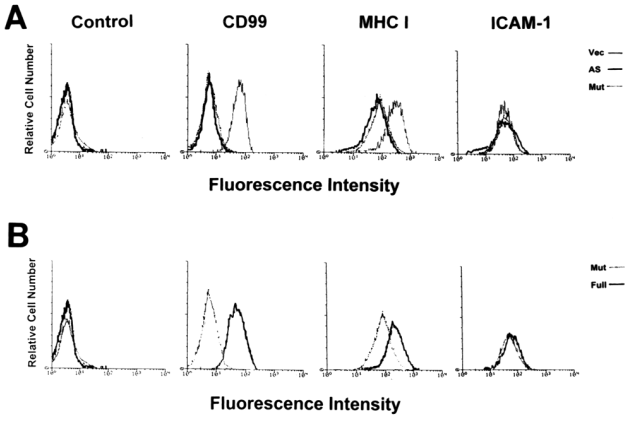

FIGURE 1. Down-regulation of the cell surface MHC class I molecules in the CD99-deficient IM-9 cells. *A*, The surface expression levels of CD99, MHC class I, and ICAM-1 were analyzed in Vec-TF (Vec), AS-TF (AS), and Mut cells by flow cytometry, as described in *Materials and Methods*. Cells were stained with each mAb and the expression level of each protein was compared. Control, isotype-matched control Ab; CD99, anti-CD99 mAb, DN16; MHC I, anti-MHC class I mAb, W6/32; ICAM-1, anti-ICAM-1 mAb, B-C14. *B*, Same as in *A* for Mut and Full-TF (Full) cells.

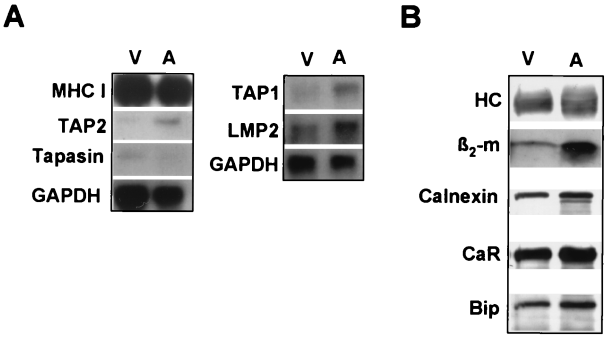

FIGURE 2. Examination of mRNA and protein expression of MHC class I subunits from Vec-TF (V) and AS-TF (A) cells. *A*, Northern blot analyses. Total RNA was extracted and analyzed, as described in *Materials and Methods*. GAPDH expression was tested as an internal control. *B*, Immunoblot analyses. Total cell lysates were detergent-extracted and 100 μg of the lysates was applied for Western blot analysis, using Abs against human MHC class I heavy chain (HC10), β_2m (BBM.1), calnexin (clone 37), CaR (rabbit polysera), Bip (10C3) as indicated. The exposure time of each immunoblot was adjusted such that all time points would be within the linear range of the film. The doublet in the calnexin blot using AS-TF cells was also observed when the Vec-TF cells were used with longer exposure, and the relationship of the minor lower band to the predominant upper band is not known.

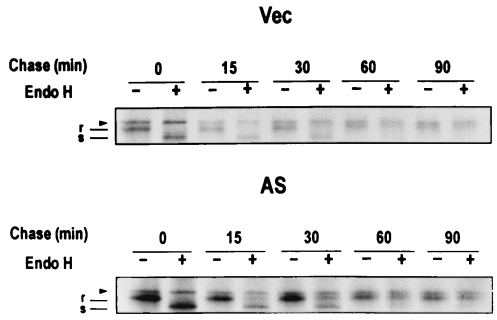

FIGURE 3. Acquisitions of endo H resistance from pulse-chase transport analyses of MHC class I molecules in Vec-TF (Vec) and AS-TF (AS) cells. Cells were pulsed for 10 min and then chased for indicated times. Lysates from pulsed and chased cells were immunoprecipitated with a mixture of mAb HC10 and W6/32, and the immunoprecipitates were digested with (+) or without (−) endo H. Unknown molecules pulled down with MHC class I molecules are shown in uppermost of each lane (▲). Letters r and s indicate the positions of endo H-resistant and endo H-sensitive forms of MHC class I heavy chain, respectively. Representative results are shown from two independent experiments.

cis- or *medial*-Golgi in AS-TF cells by endo H digestion analysis after pulse and chase (25, 26). When MHC class I molecules were digested using endo H, all of the endo H-sensitive forms of MHC class I heavy chains were converted to the endo H-resistant forms at the 90-min time point in both AS-TF and Vec-TF cell lines, indicating similar rates of conversion between the two cell lines (Fig. 3). This suggests that the transport of MHC class I molecules from the ER to the *cis*- and/or *medial*-Golgi region is not affected by CD99 deficiency.

Loss of CD99 does not affect internalization of MHC class I molecules

MHC class I molecules on the cell surfaces are constitutively internalized by endocytosis and then recycled or degraded. Recently, HIV-1 Nef protein was reported to down-regulate MHC class I molecules on the cell surface through accelerated endocytosis (16, 27). Thus, we explored whether the internalization rate of MHC class I molecules in the CD99-deficient cells is accelerated. Vec-TF and AS-TF cells were bound with W6/32 mAb at 4°C and returned to culture. At each indicated time, aliquots of cells were removed and stained with the secondary Ab, GAM-FITC, to measure the levels of uninternalized surface MHC class I molecules by flow cytometry. The proportions of W6/32-bound MHC class I complexes remained on the cell surface after a given incubation period were calculated by the percentage of the mean value at each time point relative to the mean value at zero time point, and the internalization rates seemed to be almost identical in both AS-TF and Vec-TF cell lines (Fig. 4*A*). Moreover, we confirmed the result by comparing the life spans of surface MHC class I molecules between the two cell lines after surface biotinylation. As shown in Fig. 4*B*, the half-life of surface MHC class I molecules was merely different between the two cell lines. Taken together, these results indicate that the loss of CD99 does not influence on the internalization of surface MHC class I molecules.

CD99 deficiency affects the post-Golgi trafficking of MHC class I molecules to the plasma membrane

In our previous report, the engagement of CD99 with agonistic Ab induced rapid up-regulation of MHC class I molecules in human thymocytes by accelerating the mobilization of MHC class I molecules from the cytosol to the plasma membrane (12). This finding led us to hypothesize that CD99 might function in the regulation of the approach of MHC class I molecules to the plasma membrane. To test this possibility, we quantitatively measured the amount of MHC class I molecules newly arrived on the surfaces by using FITC-conjugated W6/32 mAb after presaturating the surfaces with unconjugated W6/32 mAbs and further incubating at 37°C for a given period of time. The flow cytometric analysis clearly showed that intracellular MHC class I molecules appeared more slowly on the cell surface in the CD99-deficient cells than in the control cells (Fig. 5*A*).

To explore whether the transport of de novo synthesized MHC class I molecules was also impaired in the absence of CD99, we performed the Ab capture assay (17) after pulse and chase as described in *Materials and Methods*. In accordance with the previous flow cytometric data (Fig. 1 and Fig. 5*A*), the amounts of surface MHC class I molecules of AS-TF cells were much smaller than those of control cells (Fig. 5*B*, Vec and AS lower panels, surface), while the amounts of total MHC class I molecules in the two cell lines were almost same (Fig. 5*B*, Vec and AS lower panels, total). Newly synthesized MHC class I molecules in AS-TF cells seemed to arrive on the cell surfaces at the slower rate (Fig. 5*B*, Vec and AS upper panels). As shown in Fig. 5*C*, ~80% of newly synthesized MHC class I molecules have already been present on the plasma membrane within 60 min after the pulse in Vec-TF cells, whereas in case of AS-TF cells, much less MHC class I molecules

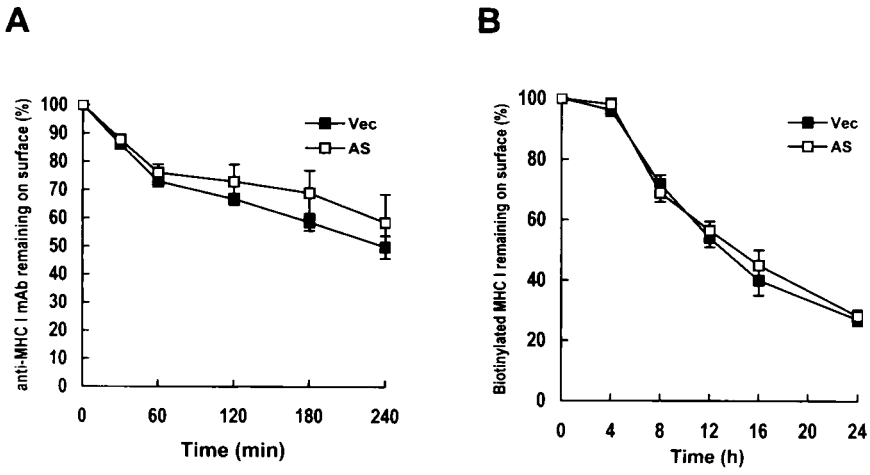

FIGURE 4. Kinetics of decrease of MHC class I molecules on the surface of Vec-TF (Vec) and AS-TF (AS) cells. *A*, Kinetics of internalization of surface-bound anti-MHC class I mAb were similar in both cell types. Cells were cultured, stained, and analyzed, as described in *Materials and Methods*. Data are the ratio of the mean fluorescence at various time points to the values obtained at time zero (200 and 400 fluorescence units for AS- and Vec-TF cells, respectively). All values are means ± SD of three separate experiments. *B*, Similar kinetics of decrease of biotinylated MHC class I molecules on the surface of Vec-TF (Vec) and AS-TF (AS) cells. The experimental procedure and quantification were performed as described in *Materials and Methods*. Data were shown as the percentage of the value at different time points relative to the values obtained at time zero. All values are means ± SD of three separate experiments.

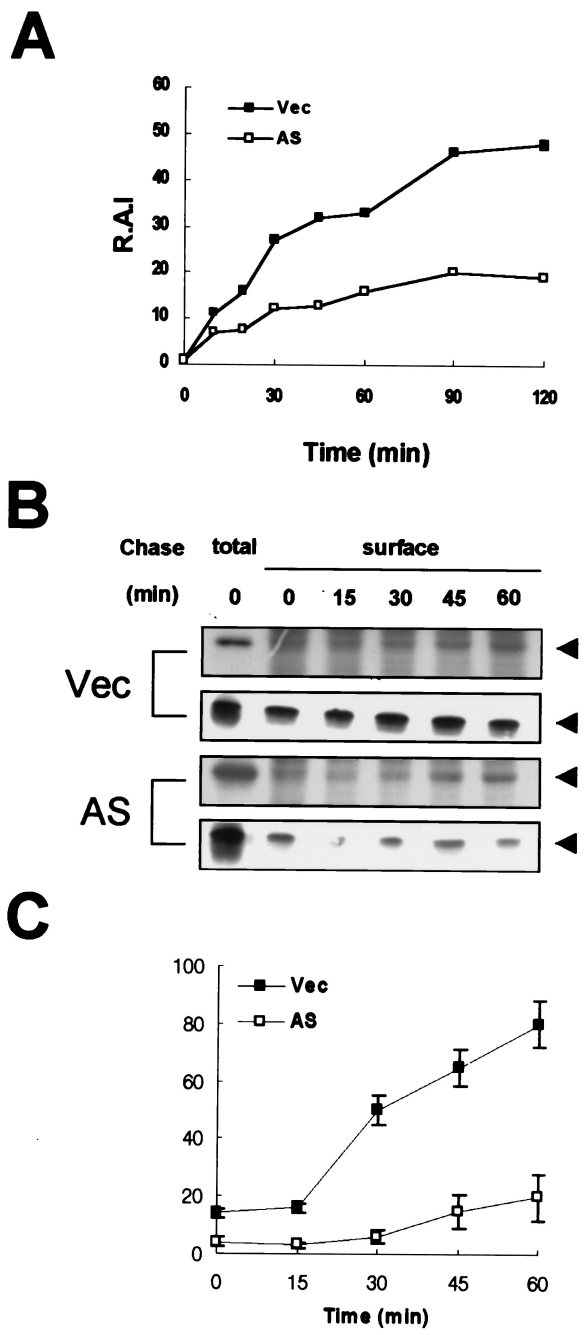

FIGURE 5. Delayed accumulations of MHC class I molecules on the surface of AS-TF cells. *A*, Measurement of cell surface arrival of MHC class I molecules in Vec-TF (Vec) and AS-TF (AS) cells using flow cytometer. The levels of the surface MHC class I molecules bound with FITC-conjugated W6/32 mAb were analyzed by flow cytometry as described in *Materials and Methods*. Relative Ag intensity was calculated by subtracting mean fluorescence values of W6/32 mAb-MHC class I complex obtained at time zero from all values at different time points and then dividing the mean fluorescence values obtained at time zero. Representative results are shown from four independent experiments. *B*, Ab captures of accumulated MHC class I molecules on the plasma membrane of Vec- and AS-TF cells. Ab capture assay was performed as described in *Materials and Methods*. Two thirds of immunoprecipitates from the Ab-captured cell lysates (surface) were directly analyzed by SDS-PAGE and autoradiography (upper autoradiographs). The remainder was analyzed by Western blotting with HC10 mAb to demonstrate that equal amounts of proteins were immunoprecipitated at every indicated time (lower autoradiographs). In the case of zero chase time point, total cell lysates without Ab capture from pulsed cells were immunoprecipitated with W6/32 mAb to ensure that newly synthesized material could be precipitated from inside the cells (total). Arrowhead (◀) indicates MHC class I heavy chain. *C*, Quantification of the kinetics of cell surface arrival of MHC class I molecules in both cells. As described in *Materials and Methods*, quantification was performed on the signal for MHC class I heavy chain. Data were shown as the percentage of the surface MHC class I molecules captured by W6/32 mAb at each time point relative to the total MHC class I molecules, which were radio-labeled and immunoprecipitated with W6/32 mAb at time zero. All values are means ± SD of three separate experiments.

(~20%) were detected on the cell surface at the same time point. Considering that total surface MHC class I molecules remain in constant levels throughout the experiment in both cell lines (Fig. 5*B*, Vec and AS lower panels, surface), the data indicate that the transport of newly synthesized class I molecules to the cell surfaces is retarded in the absence of CD99. Altogether, our results suggest that a large fraction of MHC class I molecules resides in the intracellular compartment in AS-TF cells, at least by intracellular retention of the newly synthesized class I molecules.

CD99 deficiency induces retention of MHC class I molecules in the Golgi compartment

To identify the intracellular localization of the accumulated MHC class I in the CD99-deficient cells, we performed confocal laser scanning microscopy. Cultured AS-TF and Vec-TF cells were permeabilized and stained using W6/32 mAb. As shown in Fig. 6, accumulation of MHC class I molecules in the Golgi complex was evident in AS-TF cells (Fig. 6, *A* and *B*, *panel 4*), compared with that of Vec-TF cells (Fig. 6, *A* and *B*, *panel 1*). For the detailed localization of MHC class I molecules, we performed a series of colocalization experiments of MHC class I with Golgi-resident proteins, such as α-mannosidase II and the γ subunit of AP1 adaptor complex. Anti-α-mannosidase II-specific rabbit serum (28) produced a perinuclear staining pattern characteristic of the Golgi region, consistent with a previous report (Fig. 6*A*, *panels 2* and *4*) (29) and its colocalization with MHC class I in the Golgi area was more obvious in AS-TF cells (Fig. 6*A*, *panel 6*) than in Vec-TF cells (Fig. 6*A*, *panel 3*). For the more specific localization, cells were stained with W6/32-FITC, together with Ab to γ-adaptin that is present in the *trans*-Golgi/TGN and TGN-associated vesicles (30, 31). In AS-TF cells, most of MHC class I primarily colocalized with γ-adaptin at the *trans*-Golgi/TGN albeit some traces were also found in γ-adaptin-positive vesicles known to link the *trans*-Golgi with the endocytic pathway (Fig. 6*B*, *panel 6*) (32, 33). From these results, we concluded that loss of CD99 promotes accumulation of MHC class I molecules in the Golgi, specifically, *trans*-Golgi/TGN compartment and to a much less extent, in γ-adaptin-positive vesicles.

Discussion

Recently, we reported the generation of the cells with H-RS phenotype, the morphological hallmark of HD, through forced down-expression of CD99 molecules in B cell lines (15). Interestingly, down-regulation of MHC class I molecules was synchronously observed not only in the CD99-down-regulated cells, but also in the spontaneously occurred CD99-negative cells. These facts prompted us to investigate the mechanism for the down-regulation of MHC class I molecules in the CD99-deficient B cells, because decreased expression of the cell surface MHC class I molecules is one of the important pathways for escape from host immune surveillance including H-RS cells in HD.

According to the present study, the CD99-down-regulated AS-TF cells have slight increases, if any, in the amounts of mRNA

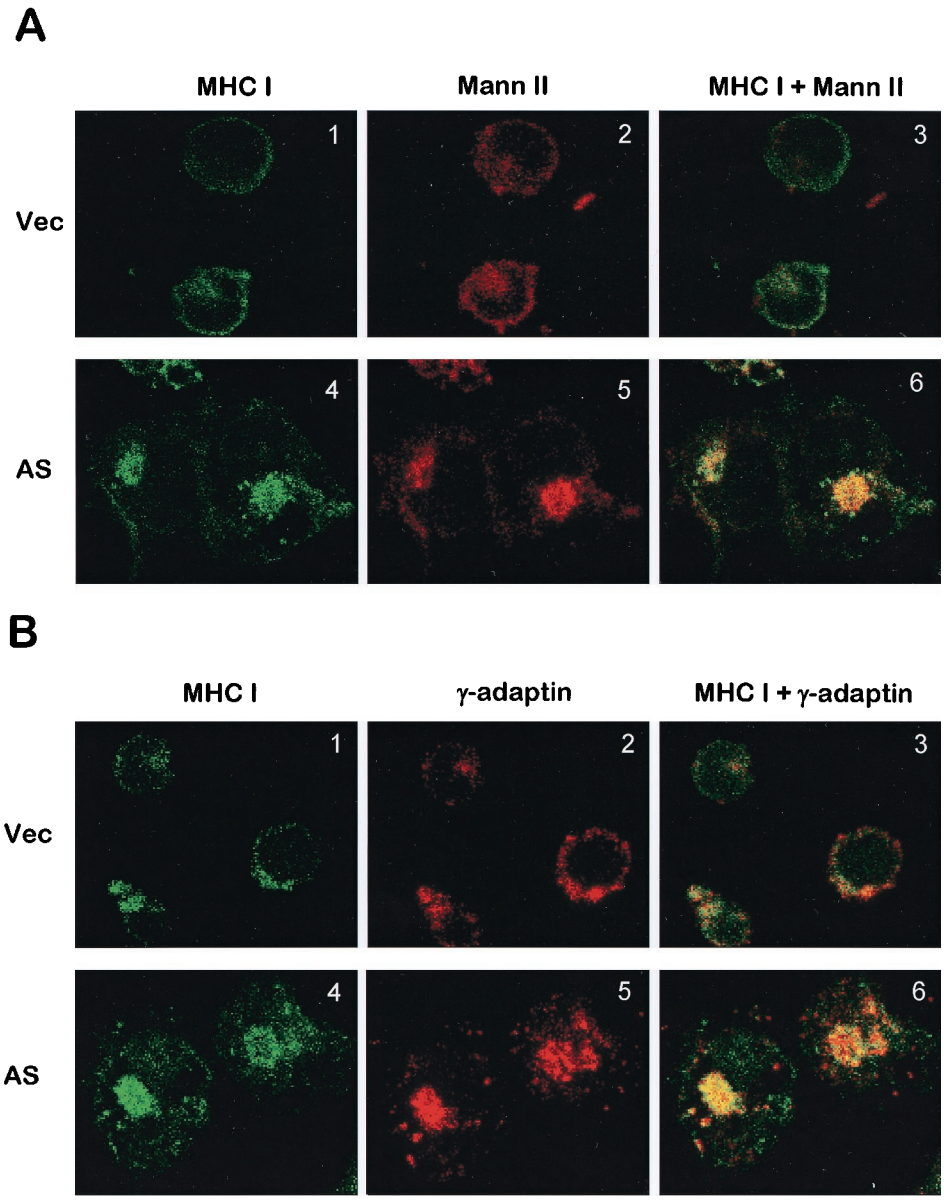

FIGURE 6. Localization of MHC class I molecules in Vec- and AS-TF cells. *A*, Colocalization of MHC class I molecules with α-mannosidase II in AS-TF cells. MHC class I was visualized by direct FITC-conjugated W6/32 mAb (*panels 1* and *4*) and α-mannosidase II by indirect fluorescence with a PE-conjugated rabbit anti-mouse IgG (*panels 2* and *5*). The overlay of two images is shown in panel 3 and 6 for Vec- or AS-TF cells, respectively. *B*, Colocalization of MHC class I complex with γ-adaptin in the *trans*-Golgi/TGN of AS-TF cells. In Vec-TF cells, MHC class I complex was rarely colocalized with α-mannosidase II (*A*, *panel 3*) and γ-adaptin (*B*, *panel 3*), as compared with those of AS-TF cells (*A*, *panel 6* and *B*, *panel 6*). Note little localization of MHC class I molecules in the γ-adaptin-positive vesicles. Both *A* and *B* show the results of original magnification ×1000. Note that AS-TF cells are much larger than Vec-TF cells.

and proteins in some molecules, especially MHC class I assembly-related molecules. To be notable, β_2m showed the most prominent increase in the translational level. The reason why these molecules are up-regulated in AS-TF cells is not certain, although it might be the IFN effect due to the double-strand RNA formed by antisense CD99 transcript (34). However, this effect is unlikely to be directly concerned with the down-expression of MHC class I molecules via CD99, because IFN has been known to induce up-regulation rather than down-regulation of MHC class I molecules on surface (35).

It was generally assumed that, upon egress from the ER, MHC class I molecules quickly reach to the cell surface without any requirement for positive sorting (10). However, recent study by Joyce (11) showed that the surface expression of MHC class I molecules was not up-regulated in the MHC class I over-expressing cell lines without defects of transport from the ER to the Golgi, suggesting that the expression of MHC class I molecules at the cell surfaces could be regulated by internalization and recycling or sorting in the *trans*-Golgi or TGN. According to several recent reports, HIV-1 Nef uses both mechanisms, acceleration of their endocytosis and accumulation in the Golgi (16, 27, 36) for the down-regulation of cell surface expression of MHC class I molecules. Thus, our present data may provide a new molecular mechanism in that CD99 regulates the expression of MHC class I molecules by altering only the transport rate from the Golgi complex to the plasma membrane without influencing endocytosis and degradation, because the newly synthesized MHC class I molecules was impeded from migrating to the plasma membrane and was accumulated in the *trans*-Golgi/TGN in AS-TF cells but not in Vec-TF cells. The finding that MHC class I molecules were primarily colocalized with γ-adaptin in the TGN, and some in γ-adaptin-positive vesicles, which mediate traffic from the *trans*-Golgi to the endosomes en route to the lysosomes (37), suggests that MHC class I molecules in AS-TF cells might be accumulated in the TGN without further lysosomal degradation. This possibility can be supported by the results of our experiments, such as no alteration in the rates of endocytosis of surface MHC class I molecules (Fig. 4*A*), in the rates of conversion from endo H sensitive to resistant forms (Fig. 3), in half-life of MHC class I molecules (Fig. 4*B*), or in restoration rates when prolonged periods of chase were performed with or without ammonium chloride, an inhibitor of lysosomal degradation (data not shown).

Because CD99 deficiency displays no effect on the MHC class I biosynthesis until the molecules reach to the *cis*-Golgi, it is likely that CD99 acts at a relatively late stage during protein transport, such as trafficking from the *trans*-Golgi/TGN to the plasma membrane. This feature allows functional distinction between CD99 molecules and other factors leading to partial or complete loss of MHC class I molecules in viral infection or malignant tumors.

During exocytosis of the cell surface and secretory proteins, the targeting of transport vesicles to the correct destination involves a large set of proteins and several layers of protein–protein interaction. Vesicular transport and targeting from one part of the cell to another require molecular motors and the actin and/or microtubule-based cytoskeletons to bring a vesicle as well as many other molecules to enhance the spatial and temporal control of membrane-trafficking events (38). Recently, it has been widely known that small GTPase families are closely related with the spatial and temporal control of exocytosis and endocytosis. For example, among the small GTPase families, CDC42 has recently been reported to control the polarized transport of secretory proteins to the basolateral plasma membrane of MDCK cells (39), as well as organization of actin and perhaps other cytoskeletal elements. Another small GTPase family, Rac1, is also known to be involved in the actin rearrangement and signal transduction (40). We previously reported that CD99 regulates the arrangement of the actin and cytoskeleton, and the CD99-mediated surface regulation of MHC class I molecules was dependent on Rac1 activity (15). In the CD99-deficient cells, the forced expression of constitutively active Rac1 led to almost the complete restoration of the level of surface MHC class I molecules. Based on these results, it could be suggested that CD99 deficiency might affect the activities of the Rho family of small GTPase, such as Rac1 (40), thus induce the defects of vesicle transport through the actin and/or microtubule-based cytoskeletons. However, because blocking of the actin polymerization by cytochalasin D treatment did not induce the down-regulation of MHC class I molecules in control cells (data not shown), CD99-dependent MHC class I regulation is unlikely to occur through actin-based cytoskeleton. In contrast, inhibition of normal Golgi function by treatments of nocodazole (microtubule disassembly inducer) (41), brefeldin A (coat protein redistribution and breakdown of the Golgi stack) (42), or wortmannin (phosphatidylinositide-3-kinase inhibitor) (43) induces down-expression of surface MHC class I molecules in control cells (data not shown). In addition, a recent report showed data suggesting that the treatment with nocodazole only affects the transport of MHC class II molecules before they leave the exocytic pathway (44). Taken together, it is highly possible that loss of CD99 may cause the stagnation of MHC class I in the *trans*-Golgi/TGN by affecting the transport of MHC class I molecules in the TGN. Therefore, it is likely that CD99 mediates the regulation of the surface expression of MHC class I molecules, by affecting the Golgi or post-Golgi trafficking. However, more detailed molecular mechanisms and related factor(s) through which CD99 regulates the post-Golgi trafficking remain to be identified.

Here, using a CD99-deficient B cell line, we showed that CD99 regulates the surface expression of MHC class I molecules by affecting the transport from the Golgi complex to the plasma membrane. Despite the fact that down-regulation of MHC class I surface expression has been observed in a significant number of HD cases (45, 46), the cellular mechanism for the down-expression has yet to be identified. Because the down-regulation of CD99 is associated with the generation of cells with H-RS phenotype, the present results might provide a possible mechanism of MHC class I down-regulation in H-RS cells of HD.

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