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Early Endosomal Rerouting of Major Histocompatibility Class I Conformers

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Major histocompatibility class I (MHC-I) molecules are present at the cell surface both as fully conformed trimolecular complexes composed of heavy chain (HC), beta-2-microglobulin (β2m) and peptide, and various open forms, devoid of peptide and/or β2m (open MHC-I conformers). Fully conformed MHC-I complexes and open MHC-I conformers can be distinguished by well characterized monoclonal antibody reagents that recognize their conformational difference in the extracellular domain. In the present study, we used these tools in order to test whether conformational difference in the extracellular domain determines endocytic and endosomal route of plasma membrane (PM) proteins. We analyzed PM localization, internalization, endosomal trafficking, and recycling of human and murine MHC-I proteins on various cell lines. We have shown that fully conformed MHC-I and open MHC-I conformers segregate at the PM and during endosomal trafficking resulting in the exclusion of open MHC-I conformers from the recycling route. This segregation is associated with their partitioning into the membranes of different compositions. As a result, the open MHC-I conformers internalized with higher rate than fully conformed counterparts. Thus, our data suggest the existence of conformation-based protein sorting mechanism in the endosomal system.

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Constitutive endocytosis of membrane components and their sorting in the endosomal system is a fundamental mechanism utilized by the cell for maintenance and regulation of membrane composition. In addition to relatively well defined clathrin-dependent and caveole-dependent pathways of endocytosis, a third pathway, which is independent of both clathrin and caveolae, constitutes a high capacity specialized pathway for regulation of membrane composition, both plasma membrane (PM) proteins and lipids, and uptake of fluid materials (Mayor and Pagano, 2007; Sandvig et al., 2008; Donaldson et al., 2009; Grant and Donaldson, 2009; Hansen and Nichols, 2009; Saftig and Klumperman, 2009). Understanding of molecular components and regulatory mechanisms of this pathway is poor, including also basic principles for its definition. Experimental evidence indicates that all three pathways share common postendocytic molecular machinery, endocytosed cargo is mixed in early endosomes (EEs), and internalized cargo is sorted either into the recycling or degradative route (Grant and Donaldson, 2009; Hansen and Nichols, 2009; Saftig and Klumperman, 2009; Jovic et al., 2010).

Irrespective of expanding research of endocytic mechanisms, a limited number of endocytic routes have been characterized. Membrane receptors internalized via clathrin-dependent endocytosis are diverged from common endosomes either into the recycling route, such as transferrin receptor (TfR) (van Dam et al., 2002), or into the degradative route, such as low-density lipoprotein (LDL) receptor (Lakadamyali et al., 2006) or epidermal growth factor (EGF) receptor (Madshus and Stang, 2009). Even though a couple of models are being discussed (Lakadamyali et al., 2006; Grant and Donaldson, 2009; Madshus and Stang, 2009; Jovic et al., 2010), the basis for their segregation in EEs is not clear. Similarly, although a considerable number of cargo proteins that use constitutive clathrin-independent internalization route have been discovered (Eyster et al., 2009; Grant and Donaldson, 2009), very little is known about their endosomal routes and principles of their sorting in the endosomal system. Apparently, in the endosomal system the decision should be made which membrane protein will be returned back to the PM and which will be directed into degradation. In contrast to clathrin-dependent cargo proteins, for which differential sorting is related to their functional path, clathrin-independent membrane cargo proteins should be subjected to sorting mechanisms that determine their concentration at the PM and establish balance with the supply from the biosynthetic pathway. In addition, endosomal membranes should distinguish misfolded, unfolded, or used membrane proteins and select them into the degradative route. Therefore, it can be expected that quality control exists also in

Abbreviations: AF, Alexa Fluor; β_2m, β_2-microglobulin; EE, early endosome; EEA1, early endosomal antigen 1; EGF, epidermal growth factor; eMHC-I, open (empty) MHC-I conformers; eL^d, empty L^d; ER, endoplasmic reticulum; fMHC-I, fully conformed MHC-I proteins; fL^d, full L^d; HC, heavy chain; IR, internalization rate; LE, late endosome; MHC-I, major histocompatibility class I; mAb, monoclonal antibody; PM, plasma membrane; Tf, transferrin; TfR, transferrin receptor; TX-100, Triton X-100.

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the endosomal system as do in the secretory pathway (Arvan et al., 2002).

In this study we used major histocompatibility class I (MHC-I) molecules, the best characterized clathrin-independent membrane cargo proteins (Mayor and Pagano, 2007; Sandvig et al., 2008; Donaldson et al., 2009; Grant and Donaldson, 2009; Hansen and Nichols, 2009), to explore whether or not conformational change of the extracellular domain affects endosomal trafficking route of a membrane protein. These molecules were used as a model system for studying protein folding in the endoplasmic reticulum (ER) and trafficking in the secretory pathway (Wearsch and Cresswell, 2008; Donaldson and Williams, 2009). MHC-I molecules reach the cell surface as fully conformed trimoleculear complexes composed of heavy chain (HC), beta-2-microglobulin (β2m), and peptide. Misfolded, incompletely assembled (free HCs), and peptide-empty MHC-I complexes are recognized by the quality control machinery that operates in the ER, retained in the ER, and translocated into cytosol for degradation by proteasomes (Wearsch and Cresswell, 2008; Donaldson and Williams, 2009). Irrespective of the stringent trafficking barrier that does not allow their exit to the Golgi, isolated HCs and non-conformed open forms in which the peptide is either loosely bound or absent (open MHC-I conformers or empty MHC-I) can be found at the cell surface (Ljunggren et al., 1990; Hansen et al., 2005; Arosa et al., 2007). Although it is not clear whether empty MHC-I can be considered as misfolded, because they have an ordered and non-denatured structure (Arosa et al., 2007), it is reasonable to expect that open MHC-I conformers are captured by a quality control mechanism that operates at the PM and in the endosomal system (Arvan et al., 2002). Moreover, such control is even more important in the endosomal system due to the inevitable conformational changes in the acidic endosomes after their constitutive uptake.

Fully conformed MHC-I complexes and open MHC-I conformers can be distinguished by well characterized monoclonal antibody reagents (Hansen et al., 2005) that recognize their conformational difference in the extracellular domain. We have shown that open conformers of murine H2-L^d molecules have a short half-life at the cell surface (Mahmutefendić et al., 2007) and segregate in EEs from their fully conformed forms (Mahmutefendić et al., 2011). Given that H2-L^d molecules have unique properties among MHC-I proteins (Hansen et al., 2005), in the present study we examined endosomal routes of fully conformed and open conformers of classical and non-classical human MHC-I alleles. We have shown that open MHC-I conformers of all alleles examined in this study are rerouted in EEs and excluded from the EE recycling route. This segregation is associated with their partitioning into the membranes of different compositions. Thus, our data suggest the existence of conformation-based protein sorting mechanism in the endosomal system.

Materials and Methods

Cell lines

Balb 3T3 fibroblasts, P815 mastocytoma, and HeLa cells were obtained from American Type Culture Collection (ATCC). L-L^d and L-D^d are cell lines that were generated by introducing the L^d and D^d gene, respectively, into murine Ltk^- DAP-3 fibroblast cells (Lee et al., 1988). J26-Cw6, J26-G1, and J26-B7 are murine Ltk-cells (H-2^k) expressing human β2m that were transfected with either HLA-Cw6 or HLA-G1 or HLA-B7 HC genes, respectively (Ferrier et al., 1985). Cells were grown in DMEM, supplemented with 10% (v/v) of fetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/ml of streptomycin, and 100 U/ml penicillin. In addition, J26 transfectans were grown with 1% pyruvate. Cell culture medium and supplements were from Gibco (Invitrogen, Eugene, OR).

Antibodies and reagents

The following monoclonal antibodies (mAbs) with reactivity to MHC-I molecules were used: 34-5-8S (mouse IgG_{2a}, ATCC HB-102) that reacts with HC of fully conformed (full) trimolecular complexes of D^d, MA-215 (mouse IgG_{2b}) that reacts with HC of full K^d (Hasenkrug et al., 1987), 30-5-7S (mouse IgG_{2a}, ATCC HB-31) that reacts with full L^d (Lie et al., 1991), 64-3-7 (mouse IgG_{2b}) that reacts with α_1 domain of free HC and distinguish peptide-empty L^d molecules (Lie et al., 1991), W6/32 (mouse IgG_{2a}, ATCC HB-95) that reacts with HC of full HLA-A, -B, -C, and -G (Dangoria et al., 2002), MEM-147 (mouse IgG_1, EXBIO, Praha, Czech Republic) that reacts with HC of full HLA-A, -B, and -C (Tran et al., 2001), MEM-G/1 [mouse IgG_1, EXBIO Praha, a.s.) that reacts with denatured HC of HLA-G (Frumento et al., 2000), and HC-10 (mouse IgG_{2a}, obtained from Dr Hidde Ploegh, Massachusetts Institute of Technology, Cambridge, MA) that recognizes peptide-empty β2m-unassociated HLA class I HCs (Stam et al., 1986). MAbs R17 217.1.3 (rat IgG_{2a}, ATCC TIB 219) recognize murine TfR. MAb reagents specific to early endosomal antigen 1 (EEA1, chicken Ig) was from Zymed (San Francisco, CA). MAb to human TfR, to the Golgi marker GM130 (mouse IgG_1), AF^{488}- and AF^{555}-conjugated EGF, transferrin (Tf), and secondary-antibody reagents to mouse IgG_{2a}, mouse IgG_{2b}, mouse IgG_1, rat Ig, and chicken Ig were from Molecular Probes (Eugene, OR). The SelectFX Alexa Fluor 488 Endoplasmic Reticulum Labeling Kit was from Molecular Probes and anti-calreticulin (mouse IgG_1) from BD Transduction Laboratories (Lexington, KY). All secondary reagents were regularly tested for cross reactivity during experiments with negative controls. Transferrin-biotin, propidium iodide, and LY294002 were from Sigma-Aldrich Chemie GmbH (Sigma-Aldrich, Tanfkirchem, Germany).

Cell surface expression and flow cytometry

Non-adherent cells and adherent cells collected by short trypsin treatment were washed in culture medium and incubated at 4°C for 30–60 min with 5 μg/ml of primary mAbs in PBS containing 10 mM EDTA, Hepes pH = 7.2, 0.1% NaN_3, and 2% FCS (PBS-A). Unbound antibodies were removed by three washes with cold PBS-A and the cells were incubated for 30 min at 4°C with 5 μg/ml of FITC-conjugated secondary antibody reagents in PBS-A. After three washes with PBS-A, cells were analyzed by flow cytometry using FACSCalibur flow cytometer (Becton Dickinson & Co, San Jose, CA). Dead cells were excluded by propidium iodide (1 μg/ml) and a total of 10,000 cells were acquired. Fluorescence signal was determined as mean fluorescence intensity (MFI) after subtraction of the background fluorescence (ΔMFI) determined on the same cells by incubation with nonreactive mAbs of the same isotype and appropriate fluorochrome-conjugated secondary reagent.

In order to determine the percentage of cell surface expressed proteins, the cells were fixed with 4% formaldehyde (20 min at r.t.) and permeabilized with 0.5% Tween (20 min at 37°C) either prior to (total cellular expression) or after (cell surface expression) incubation with primary mAbs at 4°C in PBS-A. After three washes with cold PBS-A, the cells were incubated with FITC-conjugated secondary reagent for 30 min at 4°C, and analyzed by flow cytometry as described above. The percentage of cell surface expressed molecules was calculated as $(\Delta MFI_{cell\ surface})/(\Delta MFI_{total}) \times 100$.

Immunofluorescence and confocal analysis

Cells grown on coverslips were fixed with 4% formaldehyde (20 min at r.t.) and permeabilized for either 7 min at r.t. with 0.2% Triton X-100 (TX-100) or 20 min at 37°C with 0.5% Tween. After permeabilization cells were incubated with fluorochrome-conjugated or unconjugated primary reagents for 60 min. Unbound reagents were washed with PBS and cells were either embedded or incubated for 60 min with an appropriate fluorochrome-conjugated secondary reagent. After the three washes in PBS, cells were embedded in Mowiol (Fluka Chemicals, Selzee,

Germany)-*DABCO* (Sigma Chemical Co, Steineheim, Germany) in PBS containing 50% glycerol and analyzed by confocal microscopy. Images were obtained using Olympus Fluoview FV300 confocal microscope (Olympus Optical Co., Tokyo, Japan) with 60×PlanApo objectives and either 4× or 8× zoom. Z-axis was 0.5 μm, if not indicated otherwise. Images of single cells were acquired at the same magnification, exported in a TIFF format, and processed by Fluoview, Version 4.3 FV 300 (Olympus Optical Co., Tokyo, Japan). Presentation of figures was accomplished in Adobe Photoshop (San Jose, CA).

Colocalization analysis

Colocalization events were evaluated by using a global statistic approach that performs intensity correlation coefficient-based (ICCB) analyses. We used ImageJ software, utilizing JACoP plugin (http://rsb.info.nih.gov/ij/plugins/track/jacop.html) (Bolte and Cordelières, 2006) to calculate Manders' overlap coefficients (M1 and M2). M1 is defined as the ratio of the "summed intensities of pixels from the green image for which the intensity in the red channel is above zero" to the "total intensity in the green channel" and M2 is defined conversely for red. The best-fit lower threshold to eliminate most of the signal background was determined using the threshold tool and confirmed by visual inspection. Measures were made on at least 10 cells per experimental condition. Cells were randomly selected on the same coverslip among those that were well spread and showed a well-resolved pattern. All experimental and control images were acquired using identical imaging settings.

Internalization assays

Internalization of cell surface proteins was analyzed by modified internalization assay (Coscoy and Ganem, 2000; Naslavsky et al., 2003; Mahmutefendić et al., 2007; Eyster et al., 2009) on cells detached by scraper or short trypsinization. Prior to the labeling, cells were washed three times with PBS. Cell surface proteins were labeled with specific mAb reagents (2 μg/ml) or incubated with the irrelevant antibody of the same isotype (2 μg/ml) at 4°C for 60 min. Unbound mAbs were removed by the three washes with PBS and internalization was performed at 37°C for indicated time ($t = x$ min) or cells were kept on ice (control cells, $t = 0$ min). Cell surface bound mAbs were determined by FITC-conjugated secondary reagent (5 μg/ml) and flow cytometry. To calculate the degree of internalization, the ΔMFI was calculated for each time point ($\Delta MFI_{t=x}$), representing proteins that remained at the cell surface. The ΔMFI of the control cells, which were kept on ice, represents the total cell surface expression before internalization was initiated ($\Delta MFI_{t=0}$). The percentage of internalized cell surface proteins was calculated as $(1-(\Delta MFI_{t=x})/(\Delta MFI_{t=0})) \times 100$. The internalization rate (IR) was calculated from $t_{1/2}$ value, which is determined by plotting the fraction of cell surface bound mAbs (at 4°C) that remained at the cell surface after incubation at 37°C as a function of time. First-order rate constant for internalization (k_i) was calculated as $\ln(2)/t_{1/2}$ (Ciechanover et al., 1983).

To visualize internalized proteins inside cells the internalization was performed on adherent cells grown on coverslips. Cell surface proteins were labeled with mAb reagents (2 μg/ml) at 4°C for 60 min, unbound mAbs removed by the three washes with PBS, and internalization started by addition of pre-warmed medium. Residual surface-bound mAbs were stripped by acid (1 min, pH 2.2), and internalized mAbs were visualized by immunofluorescence and confocal analysis using appropriate fluorochrome-conjugated reagent, as described above.

To visualize internalized TfR, after 60 min of starving in Tf-free medium cells were exposed to 20 μg/ml of fluorochrome-conjugated Tf at 4°C for 20 min to enable binding of conjugated Tf to TfR. Cells were then transferred to 37°C without removing Tf from medium in order to gain continuous internalization and load Tf-containing intracellular compartments.

Recycling assays

Recycling of MHC-I molecules and TfR was determined by modification of the assay as described previously (Weber et al., 2004). Briefly, cell surface proteins were labeled with mAbs at 4°C for 60 min and mAb-bound complexes internalized for 60 min at 37°C. Residual surface-bound mAbs were stripped by acid (1 min, pH 2.2), and cells were incubated at 37°C with AF^{488}-conjugated secondary antibodies to capture recycled mAb-bound complexes. To calculate the degree of recycling, the fluorescence signal was determined after recycling at 37°C by flow cytometry for each time point ($\Delta MFI_{rec,\ t=x}$) subtracted by fluorescence signal in the absence of recycling at 4°C ($\Delta MFI_{non\ rec,\ t=x}$). Cells incubated with non-binding antibodies and with AF^{488}-conjugated secondary antibodies served as negative control. Total cell surface expression, on cells that were kept on ice without internalization ($\Delta MFI_{t=0}$), and surface expression after 60 min internalization ($\Delta MFI_{int,\ t=60}$) were determined by AF^{488}-conjugated secondary antibodies. Percentage of recycled proteins was calculated as $((\Delta MFI_{rec,\ t=x} - \Delta MFI_{non\ rec,\ t=x})/(\Delta MFI_{t=0} - \Delta MFI_{int,\ t=60})) \times 100$.

TfR recycling was also quantified by detection of Tf loss form cells after pulse internalization (van Dam et al., 2002). Briefly, cells were starved in the medium without Tf for 1 h and exposed to AF^{488}-Tf (20 μg/ml) for 60 min at 37°C. Un-internalized AF^{488}-Tf was removed by washing (three times in medium with unlabelled Tf) and recycling of Tf was determined by flow cytometry as a loss of fluorescence from cells after incubation at 37°C in the medium containing 200 μg/ml of unlabeled Tf. The percentage of the recycled was calculated as follows: $(1-(\Delta MFI_{rec,\ t=x})/(\Delta MFI_{int,\ t=60})) \times 100$.

Simultaneous recycling of MHC-I proteins and TfR was determined by immunofluorescence. Cells were incubated for 90 min with mAbs to MHC-I proteins and AF-conjugated Tf (either AF^{488}-Tf or AF^{555} Tf, 20 μg/ml) at 37°C (internalization) followed by a brief acid wash (1 min, pH 2.0), and incubation with AF-conjugated secondary Abs (either AF^{555}- or AF^{488}-conjugated, respectively, 1 μg/ml) for 30–60 min (recycling). Recycling of MHC-I proteins was analyzed simultaneously to the TfR recycling on the same cell on confocal images as a loss of fluorescent signal of recycled AF-Tf and the increase of fluorescence signal of recycled mAb-bound MHC-I proteins captured by AF-conjugated secondary Abs.

TX-100 extraction of membrane molecules in live cells

Cells, grown on coverslips, were incubated with specific mAb reagents (2 μg/ml) at 4°C for 60 min. After three washes with PBS, cells were chilled on ice, treated with ice-cold 1.0% TX-100 in PBS for 1 min, fixed and processed for immunofluorescence as described above.

Statistical analysis

The experimental results were analyzed using computer applications Sigma Plot Scientific Graphing System, v8.0. Data are expressed as mean ± standard deviation (S.D.).

Results

We analyzed the expression and endosomal trafficking of MHC-I conformers of human and murine alleles on various cell lines. MHC-I conformers of classical human MHC-I proteins (HLA-A, -B and -C) were analyzed on HeLa cells by mAbs W6/32, which recognize fully conformed forms of all three alleles, and various mAb reagents that recognize open conformers (β2m unassociated HCs). In addition, we analyzed fully conformed forms and open conformers of classical MHC-I proteins HLA-B7 and HLA-Cw6 as well as non-classical allele HLA-G1 in transfected murine J26 fibroblasts. Fully conformed proteins of murine MHC-I alleles ($H2\text{-}D^d$, $H2\text{-}K^d$, and $H2\text{-}L^d$)

were analyzed with specific mAb reagents on Balb 3T3 fibroblasts, P815 mastocytoma cells, and L cells transfectants. Open conformers of murine H2-L^d were analyzed with mAb reagent 64-3-7. This reagent does not recognize open conformers of other H2 alleles (Hansen et al., 2005) and there is no mAb reagent available which distinguishes open forms of H2-D^d and H2-K^d proteins.

Distinct cellular distribution of open MHC-I conformers

Open conformers of classical MHC-I proteins (HLA-A, -B, and -C) were detected at the cell surface of HeLa cells and on murine fibroblasts transfected with either HLA-B7 or HLA-Cw6 (Fig. 1a). Open conformers of HLA-G1 were not detected on J26 transfectants (Fig. 1a), suggesting that the non-classical MHC-I proteins do not constitutively display open conformers at the cell surface. Similarly, open conformers of murine L^d molecules were expressed at the cell surface of Balb 3T3 fibroblasts, P815 mastocytoma cells, and L cells transfected with H2-L^d (Fig. 1a). At a steady state, the majority of fully conformed MHC-I proteins were found at the cell surface, whereas a majority of open MHC-I conformers were intracellular (Fig. 1b). Inside the cell, the majority of fully conformed MHC-I proteins localized in the juxtanuclear tubulo-vesicular structures, representing tubular endosomes and the Golgi, and in the perinuclear vesicles that dominated in confocal sections over the discrete punctuated or meshwork staining of the ER (Fig. 1c). On the contrary, open conformers of classical MHC-I proteins were detected in the perinuclear well shaped vesicles outside the juxtanuclear area (Fig. 1c). In some cell lines (HeLa and L-L^d), perinuclear vesicles loaded with open MHC-I conformers were concentrated in the proximity of the juxtanuclear area, but did not co-localize with TfR and the cis-Golgi marker GM130 (supplementary Fig. 1). In addition, perinuclear vesicles with open MHC-I conformers did not co-localize with ER marker proteins, excluding their ER or ER-Golgi intermediate compartment (ERGIC) origin (supplementary Fig. 2).

The well characterized endocytic route of TfR, a protein that cycle in the endosomal system via clathrin-dependent route, served as a referent route. In all cell lines the cell surface level of TfR was low (Fig. 1b) and a majority of intracellular TfR localized in the juxtanuclear tubular endosomes (Fig. 1c).

Open MHC-I conformers are internalized from the cell surface with the higher rate than fully conformed MHC-I proteins

The distinct steady state distribution suggests that open MHC-I conformers are differentially sorted in the endosomal system.

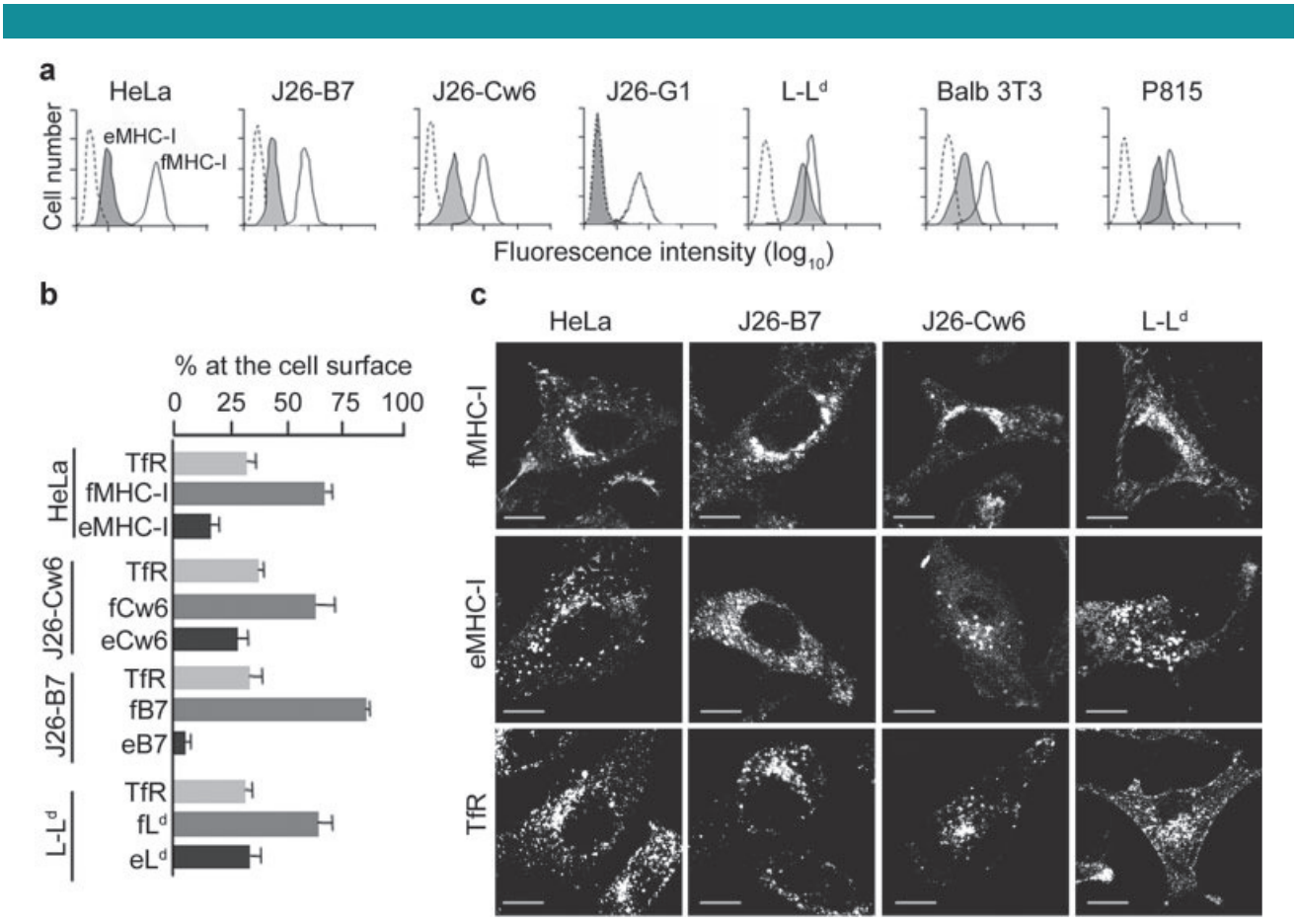

Fig. 1. Cell surface expression and intracellular distribution of open MHC-I conformers and fully conformed MHC-I proteins. a: Flow cytometric profiles of cell surface expression of fully conformed MHC-I proteins (white histograms) and open MHC-I conformers (grey histograms) at cell lines used in this study. Dashed histograms represent negative control. b: Relative cell surface distribution of fully conformed MHC-I proteins (*f*), open MHC-I conformers (e), and transferrin receptor (TfR) was determined by analysis of their expression on non-permeabilized (cell surface expression) and permeabilized (total expression) cells. The data represent the percentage of proteins detected at the cell surface ± S.D. c: Steady-state intracellular distribution of MHC-I proteins and TfR determined by confocal microscopy of permeabilized cells stained with specific primary mAb and corresponding AF^{555}-conjugated secondary Ab. Cell surface staining of fully conformed MHC-I proteins was removed by a brief acid wash. Bars, 10 μm.

Thus, we first compared kinetics of their internalization from the cell surface with fully conformed MHC-I proteins and TfR. The cell surface resident proteins were labeled with mAbs at 4°C and the loss of mAbs from the cell surface after incubation at 37°C was quantitatively followed by flow cytometry. As shown in Figure 2a, approximately half of mAb-bound cell surface resident open MHC-I conformers of all MHC-I alleles remained at the cell surface after 60 min of incubation, irrespective on cell line tested. Similar to open MHC-I conformers, approximately one-third of mAb-bound TfR was detectable at the cell surface after 60 min. At the same time, 75–85% of fully conformed MHC-I proteins still remained at the cell surface. This is accompanied with intracellular accumulation of mAb-bound proteins (see Fig. 4), indicating that the loss from the cell surface is associated with internalization of mAb-bound cell surface resident proteins. In the absence of endocytosis, i.e., in cells treated with 1-butanol or on fixed cells, bound mAbs remained at the cell surface (data not shown), indicating that the loss from the cell surface was not associated with dissociation of mAbs. These data suggest that open MHC-I conformers have different IRs than fully conformed MHC-I proteins.

In order to determine their IR, we analyzed the loss of mAb-bound cell surface proteins over time. The kinetic histogram of cell surface loss of open MHC-I conformers and fully conformed MHC-I proteins, as exemplified on HeLa cells (Fig. 2b), reveals that their loss from the cell surface follows the rule of the first-order kinetics, indicating that they are constantly internalized via the constitutive endocytic route. In contrast, internalization of mAb-bound TfR was rapid and after a mere 20 min the steady state was established at the level of 25–35%, depending on the cell line. This is consistent with the rapid TfR uptake via clathrin-dependent route and recycling, indicating the stability of mAb-TfR complexes during recycling. From the half-life of mAb-bound proteins at the cell surface we calculated their IR. The data are presented in supplementary Table 1 and summarized in Figure 2c. The IRs of open MHC-I conformers were almost an order of magnitude higher (ranging from 0.01049 min^{-1} for eL^d in L-L^d cells to 0.02233 min^{-1} in P815 cells) than IRs of fully conformed MHC-I proteins (ranging from 0.00069 min^{-1} for fD^d in Balb 3T3 cells to 0.00405 min^{-1} for fHLA-Cw6 in J26 fibroblasts). However, their IR was almost an order of magnitude lower that the IR of TfR (ranging from 0.05439 min^{-1} in P815 cells to 0.11002 min^{-1} in L-L^d cells), suggesting that open MHC-I conformers do not utilize a clathrin-dependent route for constitutive uptake.

Open MHC-I conformers segregate into distinct membrane environment at the cell surface

A higher IR of open MHC-I conformers suggests that they segregate from fully conformed MHC-I proteins along the endocytic pathway. Thus, we first tested whether or not open MHC-I conformers and fully conformed MHC-I proteins distribute in the distinct PM environment by a technique of antibody-mediated clustering (Knorr et al., 2009). Cell surface proteins were bound with specific mAbs followed by cross-linking with non-cross-reactive secondary antibodies at 4°C. Antibody-mediated clustering was initiated by short incubation at 37°C before fixation. The formed patches were analyzed at confocal sections that are tangential and orthogonal to the plane of PM. As shown in Figure 3a, the patches formed by fully conformed MHC-I proteins largely did not overlap with patches formed by open MHC-I conformers. Approximately 50–70% of patches formed by open MHC-I conformers were in the distinct localization at the PM, although in the close vicinity of fMHC-I patches (Fig. 3a). In contrast, fully conformed MHC-I proteins overlapped to the 70–80% in formed patches, as demonstrated for fK^d and fD^d on Balb 3T3 cells (supplementary Fig. 3).

Although antibody-mediated patching does not report on the environment of membrane molecules before patching, it reveals a tendency of membrane proteins to assemble into specific membrane environment (Knorr et al., 2009), such as membrane microdomains. Thus, we used cold extraction with nonionic detergent TX-100 (Schuck et al., 2003), in order to

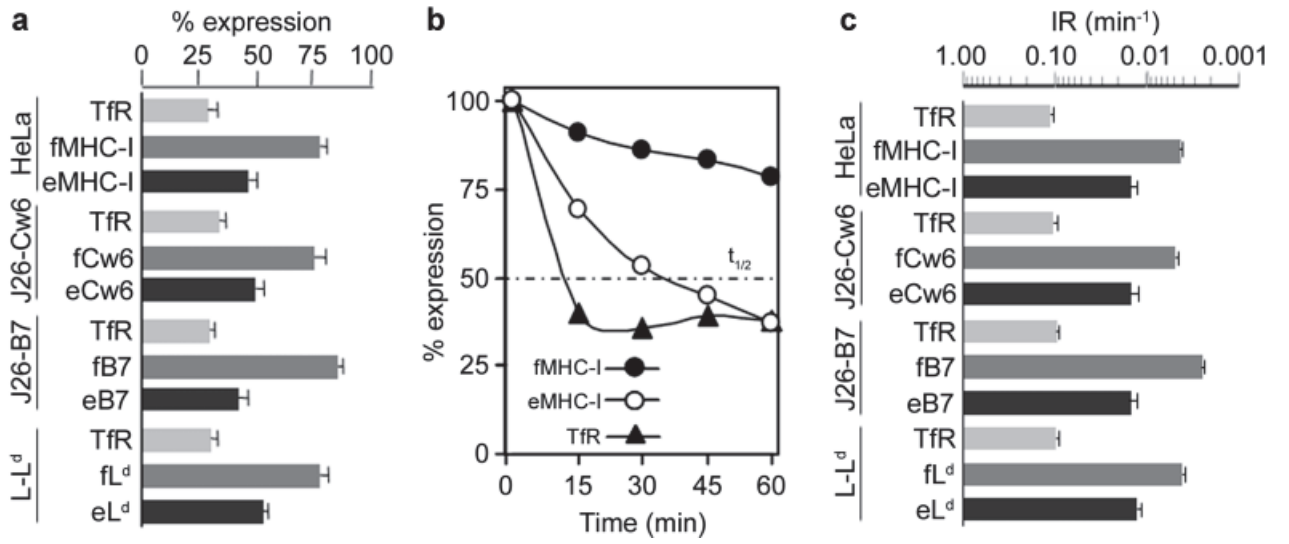

Fig. 2. Open MHC-I conformers are internalized with a higher rate than fully conformed MHC-I proteins. a: Cell surface expression of fully conformed (*f*) MHC-I proteins, open MHC-I conformers (e) and TfR after 60 min of internalization in indicated cell lines. Cells surface proteins were bound with specific primary mAbs at 4°C and incubated for 60 min at 37°C (see Materials and Methods section). Surface expression of remaining mAbs was determined by flow cytometry using FITC-conjugated secondary antibodies. Data represent the mean ± S.D. b: Kinetics of cell surface loss of mAb-bound fully conformed MHC-I proteins (fMHC-I), open MHC-I conformers (eMHC-I) and TfR determined on HeLa cells. Representative data out of five experiments are displayed. c: Internalization rates (IR) of fully conformed (*f*) MHC-I proteins, open MHC-I conformers (e) and TfR. Data represent mean values which are plotted it the logarithmic scale (log_{10}) ± S.D.

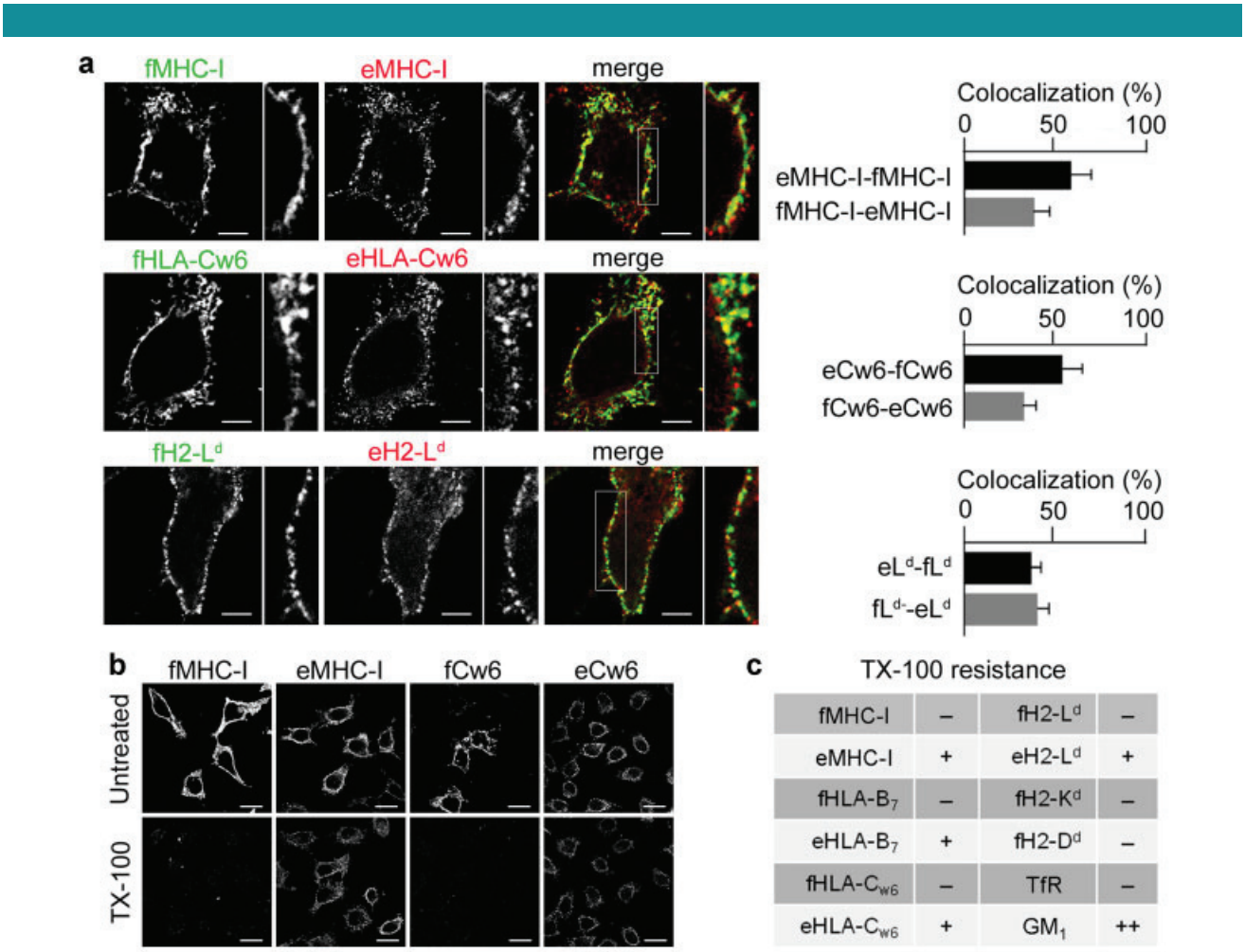

Fig. 3. Preference of open MHC-I conformers for distinct membrane environment. a: Distribution of antibody-induced patches of fully conformed MHC-I molecules (*f*) and open MHC-I conformers (e). Clusters were induced by labeling with mAbs (MEM-147 for fHLA and fCw6, HC-10 for eHLA and eCw6, 30-5-7s for fL^d, and 64-3-7 for eL^d) and appropriate non-crossreactive secondary reagent at 4°C, followed with short incubation at 37°C (5 min) prior to fixation. Quantification of colocalization is based on Manders' coefficients M1 and M2 (as described in Materials and Methods section) representing the mean ± S.D. determined on sections of 10 cells in three independent experiments that are tangential and orthogonal to the plane of the plasma membrane. Bars, 5 μm. b: Confocal images of HeLa and J26-Cw6 cells with cell surface mAb-bound MHC-I molecules after exposure to cold TX-100 prior to staining with AF^{555}-conjugated secondary antibodies. Bars, 20 μm. c: Summarized TX-resistance of cell surface fully conformed MHC-I proteins (*f*), open MHC-I conformers (e), TfR and ganglioside M1 (GM1). (++), >70% cells positive after cold TX-100 treatment with unchanged fluorescence intensity; (+), >70% cells positive after cold TX-100 treatment with reduced fluorescence intensity; (−) >90% cells negative after cold TX-100 treatment. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

examine whether or not open MHC-I conformers localize in lipid-disordered or lipid-ordered membrane composition. The cold TX-100 treatment, at the concentration that washed out TfR (Fig. 3c) (Fivaz et al., 2002), but not GM_1 (Fig. 3c) (Hullin-Matsuda and Kobayashi, 2007), completely washed out fully conformed human and murine MHC-I proteins from the cell surface of all cell lines used in this study (Fig. 3b,c). This is consistent with several reports demonstrating that fully conformed MHC molecule partition in lipid-disordered phase at the cell surface (Naslavsky et al., 2004; Knorr et al., 2009). However, open MHC-I conformers remained at the cell surface after cold TX-100 treatment (Fig. 3b,c), indicating that they have preference for lipid-organized detergent-resistant membrane composition. The cold TX-100 extraction completely washed out unbound as well as patched fully conformed MHC-I proteins but not open MHC-I conformers from the cell surface (data not shown), indicating that mAb binding or patching do not change their microdomain localization. Thus, our data suggest that the starting point for endocytosis of open MHC-I conformers is distinct, implicating the distinct sites of endocytic entry.

Internalized open MHC-I conformers segregate in perinuclear early endosomes from fully conformed MHC-I proteins and clathrin-dependent cargo

We next analyzed trafficking of internalized open MHC-I conformers by monitoring of cell surface mAb-bound proteins (Naslavsky et al., 2003; Naslavsky et al., 2004; Eyster et al., 2009; Knorr et al., 2009). Binding of antibodies did not alter their trafficking and did not cause cross-linking (data not shown), as also described by others (Eyster et al., 2009). In addition, unspecific mAbs of the corresponding isotypes did not stain intracellular structures (data not shown), indicating that the fluid phase uptake of mAbs did not contribute to the staining signal.

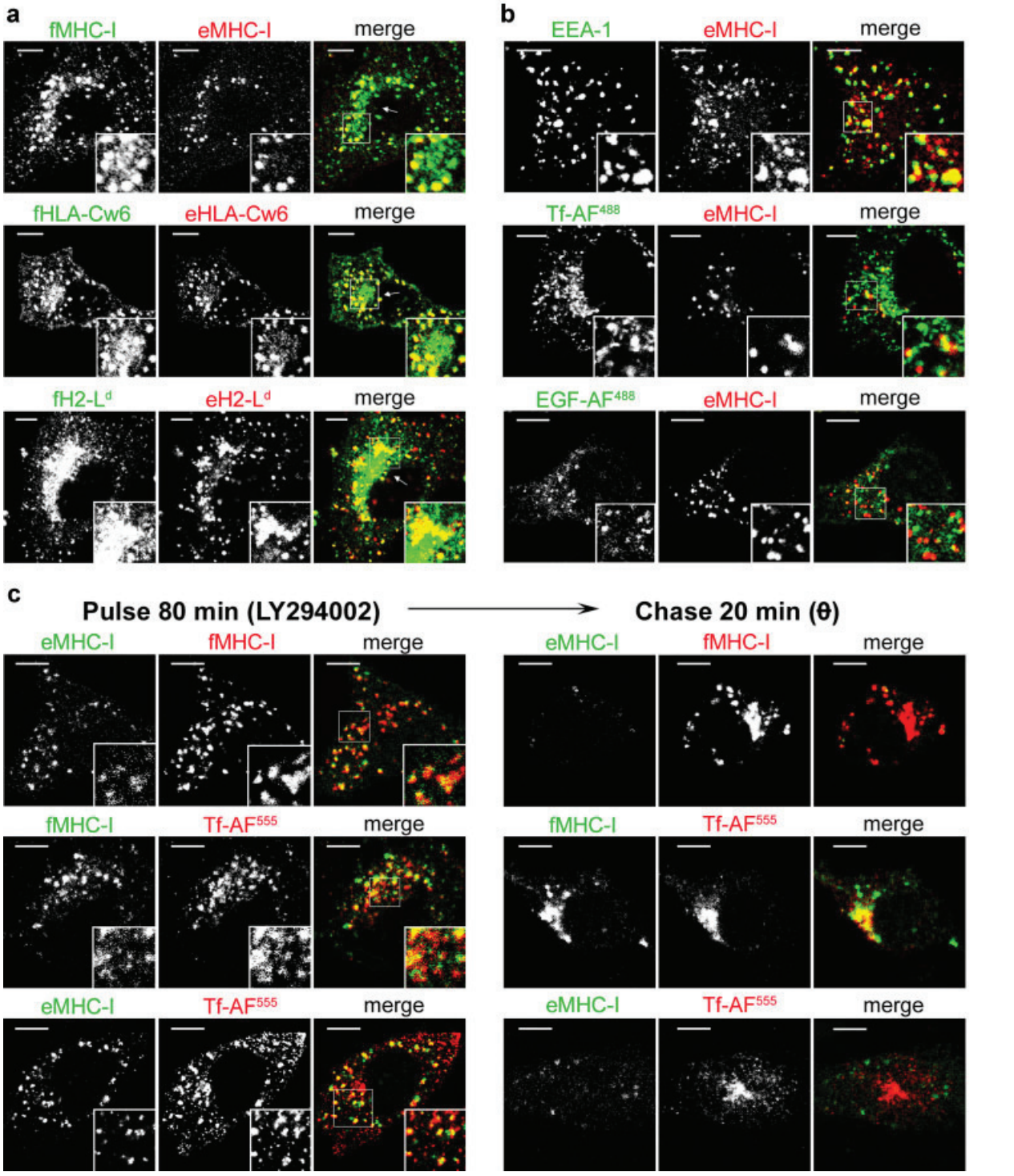

Fig. 4. Internalization pattern of open MHC-I conformers and fully conformed MHC-I proteins. a: Co-internalization of open MHC-I conformers (*e*) and fully conformed MHC-I proteins (*f*) in HeLa, J26-Cw6 and L-L^d cells. Cell surface MHC-I were labeled with mAbs (MEM-147 for fHLA, HC-10 for eHLA, 30-5-7s for fL^d, 64-3-7 for eL^d) at 4°C and internalized at 37°C for 60 min. Arrows point to juxtanuclear tubular endosomes. b: Co-localization of internalized mAb-bound open MHC-I conformers of HeLa cells (60 min internalization) with EEA1, and co-internalized Tf-AF^{488} and EGF-AF^{488}. c: Co-internalization (80 min) of mAb-bound open MHC-I conformers of HeLa cells with mAb-bound fully conformed MHC-I proteins and Tf-AF^{555} labeled TfR in the presence of 100 μM LY294002 (pulse) and their redistribution after 20 min incubation without LY294002 (chase). Bars: 5 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

We monitored intracellular trafficking of mAb-bound MHC-I proteins at various times after endocytic uptake and observed that after 60 min the staining pattern which corresponds to steady state distribution was established. At that time, in all cell lines, open MHC-I conformers co-localized with internalized fully conformed MHC-I proteins in vacuolar perinuclear endosomes surrounding the juxtanuclear area (Fig. 4a). However, in contrast to the fully conformed MHC-I proteins (supplementary Fig. 4), open MHC-I conformers did not localize in the juxtanuclear tubular endosomes (Fig. 4a, indicated by arrows). A part of perinuclear endosomes loaded with open MHC-I conformers, as well as with fully conformed MHC-I proteins, was positive for early endosomal antigen1 (EEA1) and internalized TfR (Fig. 4b), two markers of EEs, suggesting their early endosomal localization. Similarly, a part of open MHC-I conformers colocalized with internalized EGF receptor (Fig. 4b), a clathrin-dependent cargo protein that travels into LEs via dynamic EEs (Lakadamyali et al., 2006), suggesting that a part of open MHC-I conformers after 60 min of internalization reached LE intermediates. Altogether, these data indicate that internalized open MHC-I conformers segregate from fully conformed MHC-I proteins and clathrin-dependent cargo in static EEs during their maturation into LE intermediates.

To further clarify the sorting point of MHC-I conformers, we performed pulse internalization in cells treated with LY294002, a reversible inhibitor of phosphatydil-inositol-3-kinase (PI3K) which arrests progression from EEs toward LEs and the juxtanuclear recycling compartment (Lindmo and Stenmark, 2006). In HeLa cells (pulse at Fig. 4c), LY294002 treatment retained open MHC-I conformers and fully conformed MHC-I proteins in perinuclear endosomes together with TfR, suggesting that PI3K inhibition arrests progression toward the endo-lysosomal route. Fully conformed MHC-I and TfR localized in perinuclear vesiculo-tubular endosomes (Fig. 4c), but did not concentrate in typical juxtanuclear tubulo-vesicular compartment (compare with Fig. 4a,b). After the lift of PI3K inhibition (chase at Fig. 4c), fully conformed MHC-I proteins and TfR rapidly proceeded into the juxtanuclear area, whereas open MHC-I conformers were not detectable on confocal images after 20 min of chase. These data support our previous conclusion that open MHC-I conformers segregate in EEs and proceed into the rapid late endosomal degradation route, whereas TfRs and a fraction of fully conformed MHC-I proteins proceed into the recycling route (see Fig. 5b,e).

Essentially the same pattern as in HeLa cells was observed with human MHC-I proteins transfected into murine J26 fibroblasts (data not shown). However, open L^d conformers, in addition to their entry into the degradative route, were retained in late endosomal intermediates (Mahmutefendić et al., 2011). This suggests additional unique property of murine open L^d conformers.

Open MHC-I conformers are excluded from early endosomal recycling routes

Absence of open MHC-I conformers from the juxtanuclear tubular endosomes suggests for their exclusion from the slow recycling route. Thus, in order to test whether open MHC-I conformers can recycle, we pulse internalized mAb-bound proteins and captured recycled complexes at the cell surface with AF^{488}-conjugated secondary antibodies (Weber et al., 2004). With this assay we were able to capture ~80% of internalized mAb-TfR (Fig. 5a), which is consistent with observations on other cell lines (van Dam et al., 2002) and with the recycling efficiency determined by the assay based on the loss of Tf-AF^{488} from the cells (Fig. 5f). In contrast to TfR, approximately 25–30% of internalized mAb-bound fully conformed MHC-I proteins recycled whereas open MHC-I conformers were not detected by the recycling assay at all (Fig. 5a), indicating that open MHC-I conformers cannot recycle.

Given that open MHC-I proteins might distribute in various endosomal intermediates after internalization, including those that cannot form recycling membranes, we internalized mAb-bound proteins in the presence of PI3K inhibitor LY294002, which arrests open MHC-I conformers in EEs together with internalized TfR (Fig. 5b). Upon the lift of PI3K inhibition, the majority of TfR recycled, which is demonstrated as a loss of AF^{555}-Tf fluorescence from cells (Fig. 5b) and quantitative analysis by flow cytometry (Fig. 5c). The same was observed also for fully conformed MHC-I (Fig. 5c). This suggests that after the reversible PI3K inhibition, the recycling cargo can be directed into the recycling route. Under the same conditions, open MHC-I proteins were not detected to recycle (Fig. 5b). Similar result was achieved also when TfR and MHC-I proteins were internalized at 16°C, a temperature that arrests early endosomal maturation at an earlier stage than PI3K inhibition (van Dam et al., 2002). Namely, upon the shift to 37°C, TfR fully recycled whereas fully conformed MHC-I proteins recycled with a reduced efficiency (Fig. 5d). In contrast, recycled open MHC-I conformers were not detected (Fig. 5d). Taken collectively, these data indicate that open MHC-I conformers are unable to reach the recycling domain of EEs.

The inability of early endosomal recycling of open MHC-I conformers reveals a discrepancy between their IR, which is much lower, and the rate of constitutive uptake of the PM (0.069–0.138 min^{-1}) (Hao and Maxfield, 2000), which should equal their endocytic rate. Similarly, although fully conformed MHC-I proteins recycle, the rate of their recycling cannot balance the constitutive PM uptake, which is much faster. This suggests an additional rapid return mechanism that balances fast uptake of PM prior to entry of endocytosed MHC-I proteins into EEs. To address this, we loaded cells with mAb-bound TfR and MHC-I proteins, removed un-internalized mAbs from the cell surface by a brief acid wash, and exposed cells to fluorochrome-conjugated secondary reagents for 30 min, immediately after the acid wash and 30 or 60 min later (Fig. 5e). The majority of recycled mAb-TfR was detected in perinuclear early endosomal vacuolar intermediates, whereas the majority of recycled fully conformed MHC-I proteins was found at the PM and in subplasmalemal intermediates, even when secondary reagents were added one (Fig. 5e) or two (see supplementary Fig. 5 for complete experiment) hours after the acid wash. The subplasmalemal intermediates loaded with recycled MHC-I proteins were accessible to acid (supplementary Fig. 5) suggesting for their cycling in pre-early endosomal tubular membrane invaginations (Massol et al., 2005). This indicates that the majority of recycled MHC-I proteins remains in their subplasmalemal pre-early endosomal recycling route. However, we were unable to detect open MHC-I conformers by the same assay, presumably because capturing of recycled complexes from EEs and juxtanuclear recycling endosomes is critical for the sensitivity of the assay.

In order to support our conclusion that all constitutively internalized MHC-I proteins cycle prior entry into EEs, we internalized mAb-MHC-I in cells treated with tetrafluoraluminate (AlF_4^-), an inhibitor of endosomal recycling (Sheff et al., 1999), which almost completely inhibited recycling of TfR and full MHC-I proteins in L-L^d cells (Fig. 5f). Under the same experimental conditions AlF_4^- rapidly reduced the cell surface level of both fully conformed MHC-I and open MHC-I conformers (supplementary Fig. 6), respectively, and increased their IR for 1.5–2-fold (Fig. 5g). Given that open MHC-I conformers do not recycle via EEs, sensitivity to AlF_4^- suggests the existence of a recycling route of open MHC-I conformers, presumably the same pre-early endosomal subplasmalemal route used by fully conformed MHC-I proteins.

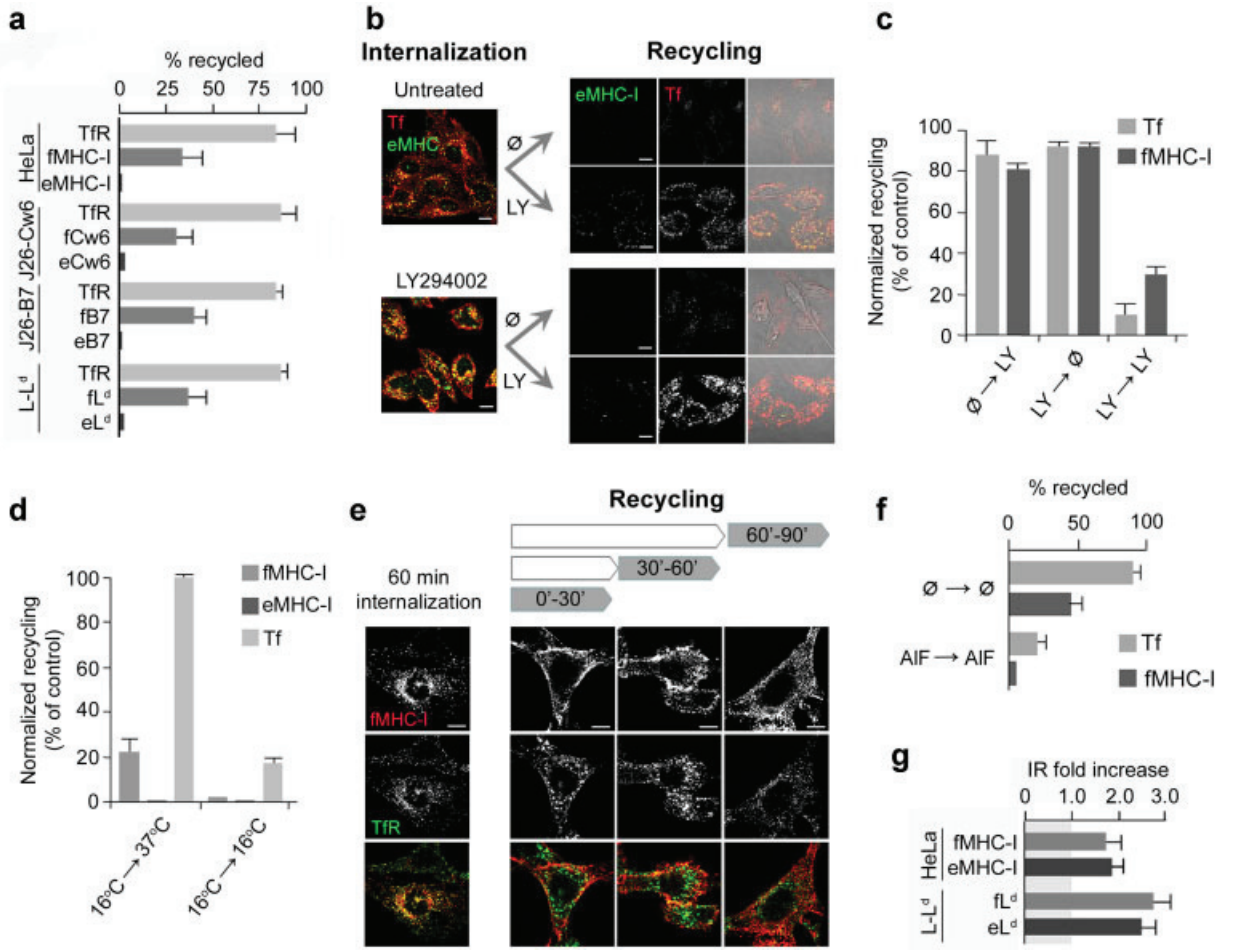

Fig. 5. Open MHC-I conformers are excluded from early endosomal recycling routes. a: Percentage of recycled TfR, fully conformed MHC-I (*f*) and open MHC-I conformers (e) after 60 min loading of endosomal compartments (see Materials and Methods section). Data represent the mean ± S.D. from five experiments. b: Recycling of TfR and open MHC-I conformers from early endosomes of HeLa cells. mAb labeled open MHC-I and AF^{555}-Tf labeled TfR were internalized 90 min in untreated or LY294002 treated (100 μM) cells. After the brief acid wash, the cells were incubated 60 min with AF^{488}-anti mouse IgG_{2a} (recycling) in the absence (Ø) or presence (LY) of the inhibitor. Bars, 10 μm. c: Effect of LY294002 (100 μM) on recycling of TfR and fMHC. HeLa cells were 90 min incubated with AF^{488}-Tf or mAb W6/32 in the absence (Ø) or presence (LY) of the inhibitor. After the short acid wash, the cells were incubated 60 min (recycling) with AF^{488}-anti mouse IgG_{2a} in the absence (→Ø) or presence (→LY) of the inhibitor. Data represent mean percentage (± S.D.) of recycled normalized to the recycled in untreated cells. d: Recycling of TfR, fully conformed MHC-I and open MHC-I conformers from 16°C early endosomes. HeLa cells were loaded with AF^{488}-Tf or mAb labeled MHC-I proteins (90 min) at 16°C, acid washed, and incubated with AF^{488}-conjugated secondary Ab reagents (60 min), either at 16°C or at 37°C. Data represent mean percentage (± S.D.) of recycled normalized to the recycled in cells at 37°C. e: Subplasmalemal recycling of MHC-I proteins. IFN-gamma treated Balb 3T3 cells were incubated 60 min with mAbs R17 and 34-5-8s at 37°C (60 min internalization), acid washed (pH 2.0 for 1 min) and intracellular mAb-bound TfR and D^d were visualized on permeabilized cells with AF^{488}-anti rat IgG and AF^{555}-anti mouse IgG_{2a}, respectively. After the acid wash, the cells were incubated in the fresh medium at 37°C which was replaced with medium containing AF^{488}-anti rat IgG and AF^{555}-anti mouse IgG_{2a} in the corresponding periods of incubations. After 30 min of incubation with secondary reagents, the cells were either fixed or briefly acid washed prior confocal analysis. Bars, 10 μm. f: Effect of AlF_4^- on recycling of TfR and fMHC-I. $L\text{-}L^d$ cells were either loaded with Tf-AF^{488} or with fL^d labeled with mAb 30-5-7s in the presence of AlF_4^- (30 mM NaF + 50 μM $AlCl_3$), acid stripped and recycling in untreated cells (Ø→ Ø) and in the presence of AlF_4^- (AIF→AIF) quantified by flow cytometry. Data represent the mean ± S.D. from three experiments. g: Internalization rate (IR) of fully conformed MHC-I (*f*) and open MHC-I conformers (e) in cells treated with AlF_4^- for 60 min. Data represent the fold increase in IRs relative to untreated cells. Shown are the means ± S.D. from three to six experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

Discussion

Irrespective to the stringent quality control in the ER, a significant fraction of MHC-I proteins of all classical MHC-I alleles is displayed at the cell surface in their open forms. Although their biogenesis at the cell surface remains unclear (Gromme et al., 1999; Dunbar and Ogg, 2002), the existence of open MHC-I conformers points to their significant physiological role in the maintenance of MHC-I homeostasis in the endomembrane system and in the process of exogenous antigen presentation. In this study we took the opportunity of having appropriate mAb tools, which distinguish the same proteins with conformational difference in the extracellular domain, to test the hypothesis that PM and endosomal membranes are able of distinguishing non-conformed proteins and therefore redirect their trafficking, as established for membranes of the ER (Wearsch and Cresswell, 2008; Donaldson and Williams, 2009). We demonstrated that PM and endosomal membranes distinguish open MHC-I conformers and displace them from the route of constitutive endosomal

trafficking used by fully conformed MHC-I proteins. Thus, in addition to the well known principle of protein sorting which is based on the recognition of cytoplasmic domain targeting sequences by adaptor proteins and sorting into the clathrin-coated pits (Smart et al., 1999; Dell'Angelica, 2001) and in endocytic carriers (Traub, 2003), we demonstrate that membrane proteins that lack adaptor protein-targeting sequences, such as MHC-I molecules (Eyster et al., 2009), may be sorted by a mechanism that distinguishes conformational change in the extracellular/luminal domain. Thus, studies of endosomal trafficking of fully conformed MHC-I proteins and open MHC-I conformers provide evidence for conformation-based sorting in the endosomal system and reveal a basic principle of protein sorting in the endosomal system.

Monoclonal antibody reagents to open conformers of classical HLA proteins recognize unassembled HCs (Schnabl et al., 1990; Perosa et al., 2003; Hansen et al., 2005) whereas 64-3-7 reagent used for L^d molecules recognizes also HCs associated with β2m (Hansen et al., 2005). Thus, the similar behavior of murine L^d molecules and human MHC-I proteins indicate that the opening of the peptide-binding groove, a relatively small unfolding event, is sufficient to redirect trafficking of open MHC-I conformers. In addition, our studies demonstrate that association with β2m is not sufficient for the sensory system in cellular membranes to consider HC-β2m complexes as folded form.

The antibody-mediated clustering assay and the resistance to a non-ionic detergent (Fig. 3) demonstrate that open MHC-I conformers segregate from their fully conformed counterparts already at the cell surface by partitioning into distinct membrane microenvironment. Fully conformed MHC-I proteins partition into nonionic detergent soluble membrane composition, which is consistent with observations in other studies (Naslavsky et al., 2004; Knorr et al., 2009), whereas open MHC-I conformers partition into Triton X-100 resistant sphingolipid-rich (Mahmutefendić et al., 2011) membrane composition. Such, distinct, partitioning may represent a basis for their distinct sorting into nascent endocytic carriers during constitutive uptake of the PM. Constitutive uptake, which exists in all cell types, occurs independently on clathrin and dynamin (Naslavsky et al., 2004; Donaldson and Williams, 2009) and internalizes the equivalent of the entire PM several times in an hour (Steinman et al., 1983; Hao and Maxfield, 2000; Grant and Donaldson, 2009). Constitutive membrane invaginations, which lead either to vesicle or tubule formation, are heterogeneous regarding membrane lipid composition, connection to the actin network and engagement of various small GTPases (Mayor and Pagano, 2007; Sandvig et al., 2008; Donaldson et al., 2009). Therefore, the segregation of open MHC-I conformers into highly lipid-organized parts of the PM will inevitably lead to their recruitment into distinct membrane invaginations and, thereby, to distinct regulatory requirements for their constitutive uptake, and perhaps a distinct endocytic rate.

Constitutive IR of fully conformed MHC-I proteins (~0.0007 to ~0.0035 min^{-1}) is much lower than the constitutive uptake of PM, which occurs with the constant rate of 0.069–0.138 min^{-1} (Hao and Maxfield, 2000), suggesting that a low IR is maintained with their high recycling rate and recycling efficiency. Although the rate of recycling of fully conformed MHC-I protein is similar to the recycling rate of TfR (~0.07 min^{-1} in HeLa and L-L^d cells, data not shown), their recycling efficiency is rather low, which is consistent with an observation using different assay (Naslavsky et al., 2004). Thus, in contrast to TfR, the low recycling efficiency cannot balance the rate of constitutive membrane uptake. In this report we demonstrate that a significant fraction of recycled fully conformed MHC-I proteins remains in the subplasmalemal rapid recycling route, presumably acid-accessible short dynamic tubular intermediates and deep tubular invaginations (Massol et al., 2005), which can explain the low constitutive IR of MHC-I proteins.

In contrast to full MHC-I, our data demonstrate that open MHC-I conformers are excluded from early endosomal recycling routes. Thus, although their IR (~0.01 to ~0.022 min^{-1}) can be almost 10- higher than IR of fully conformed MHC-I proteins, it is still much lower than the constitutive uptake of the PM. This would imply their substantially different uptake pathway or their maintenance at the cell surface by the direct return. Although the difference in the endocytic uptake cannot be excluded, their increased rate after AlF_4^- treatment, which inhibits recycling in the entire endosomal system (Radhakrishna and Donaldson, 1997; Hana Mahmutefendić, Gordana Blagojević Zagorac, and Pero Lučin, unpublished data), suggests that a substantial fraction of constitutively endocytosed open MHC-I conformers is rapidly returned to the PM, as fully conformed MHC-I proteins. However, we were unable to directly demonstrate open MHC-I conformers in the subplasmalemal recycling route.

Regardless of a way of constitutive endocytic uptake, fully conformed MHC-I proteins and open MHC-I conformers meet with clathrin-dependent cargo in "classical" EEs, as defined on HeLa cells by the presence of Rab5 and phosphatidylinositol 3-phosphate (PI3P) and subsequent binding of EEA1 (Donaldson et al., 2009; Jovic et al., 2010). Progression and segregation of fully conformed MHC-I proteins and open MHC-I conformers requires membrane lipid composition that is generated by phosphatidylinositol 3-kinase (PI3K) since reversible inhibition of PI3K with LY294002 (van Dam et al., 2002; Naslavsky et al., 2003) arrest open MHC-I conformers and fully conformed MHC-I proteins in perinuclear endosomes, together with clathrin-dependent cargo (Fig. 4). After removal of LY294002, TfR and a fraction of fully conformed MHC-I proteins are rapidly translocated into juxtanuclear tubular endosomes, while open MHC-I conformers are not detectible after 20 min, suggesting for their rapid entry into lysosomes of HeLa cells. Therefore, we can conclude that inability of progression into the EE recycling domain is critical for sorting of open MHC-I conformers. This argues against the concept that cargo without specific cytoplasmic signal is targeted by default into the tubular endosomal network and recycling (Saftig and Klumperman, 2009). A majority of full MHC-I and all open MHC-I conformers that reach vacuolar EE remain in them and travel the degradative route. This is line with the conclusion that protein down-regulation can be accomplished by a loss in recycling efficiency without the need of a specific cytoplasmic tail sorting signal and that lysosomal targeting may reflect the biophysical properties of the proteins themselves in the sorting endosome (Zaliauskiene et al., 2000).

The exclusion of open MHC-I conformers from recycling tubules can be explained by their preference for lipid-organized membrane environment and their propensity of oligomerization. Although, fully conformed MHC-I proteins also form clusters at the cell surface (Catipovic et al., 1994; Matkò et al., 1994; Pfeffer, 2003), open MHC-I conformers have a much higher propensity of oligomerization (Capps et al., 1993; Matkò et al., 1994; Arosa et al., 2007). Conformational change that occurs after dissociation of peptide or β2m from HC of MHC-I molecules, particularly in the acidic environment of EEs, can facilitate oligomerization of open conformers, as demonstrated by the enhanced oligomerization of TfR after modification of the extracellular domain (Jenei et al., 1997). Hyperoligomerzation of open MHC-I conformers may assist their partitioning into the lipid-organized composition and constrain their segregation into tiny recycling tubular endosomes. Namely, constraining in the spherical part of sorting endosomes and partitioning into endosomal inward invaginations has been proposed as sorting mechanism in the

endosomal system (Hao et al., 2004). This conclusion can be supported with our observation that endosomal sequestration of open MHC-I conformers requires proper endosomal acidification (Mahmutefendić et al., 2011) and PI3P rich membrane composition (Fig. 4), which is essential for endosomal inward invagination (Gu and Gruenberg, 1999; Kooijman et al., 2003; Lindmo and Stenmark, 2006). Given that it is now clear that there is heterogeneity of lipid-organized domains (Smart et al., 1999; Pfeffer, 2003), it cannot be excluded that open conformers partition into lipid-organized domains with higher degree of order, which cannot bud into recycling tubular extensions. Thus, additional characterization of membrane domains is required in order to prove lipid-based segregation of open MHC-I conformers in endosomes.

In conclusion, the resolution of endosomal trafficking of open MHC-I conformers is important for understanding the exogenous antigen presentation pathway and development of vaccination strategies. Open MHC-I conformers may represent a pool of MHC-I molecules for exogenous peptide loading in LEs and target for potential loading by designed peptide vaccines which can be delivered in LE by appropriate carriers. Additionally, their segregation in the endosomal system also points to the fundamental principles of protein sorting in endomembranes. Indeed, MHC-I molecules helped us in understanding basic principles for sorting of unfolded or unassembled proteins in the secretory pathway, and quality control machinery that operates in the ER has been characterized by using MHC-I molecules (Wearsch and Cresswell, 2008; Donaldson et al., 2009). Their biological properties of conformational variability, molecular interactions that are well resolved and availability of reagents that precisely distinguish various forms make MHC-I molecules a useful tool for characterization of protein sorting principles and quality control in the endosomal pathway as well.

Ethical Standards

The authors declare that the experiments comply with the current laws of the Republic of Croatia.

Acknowledgments

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