OPEN MHC-I CONFORMERS RECYCLES VIA LATE ENDOSOMAL RECYCLING ROUTE

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Introduction:

MHC class I molecules are complexes of heavy chain (HC) noncovalently associated with the peptide and β_2-microglobuline. Conformational change in the extracellular domain of HCs leads to their disassembly and formation of open conformers, a process that normally occurs in normal cells and results in their presence at the cell surface. Open MHC-I segregate from fully conformed MHC-I proteins at the cell surface and travel late endosomal (LE) route after constitutive endocytic uptake.

Endosomes represents very complex network of diferrent microdomains and subcompartments where endocytosed molecules are segregated and processed.

The aim of our study was to characterize trafficking route of open L^d molecules (empty L^d) in murine fibroblast-like cells, and to understand the possibility of their recycling from late endosomal compartments.

Results:

I. DISTRIBUTION OF OPEN L^d CONFORMERS IN LATE ENDOSOMES

1. Open L^d conformers are sorted into degradative route

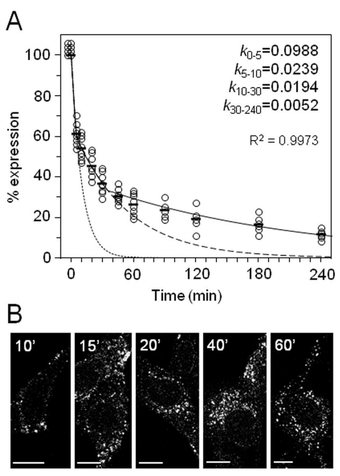

Figure 1. **Internalization kinetics of cell surface empty L^d molecules.**
(**A**) *Cell surface loss of mAb-bound eL^d molecules (mAb:eL^d) in Balb 3T3 cells*. Cell surface eL^d were labeled with 64-3-7 mAbs at 4ºC for 60 min and remaining mAb:eL^d at the cell surface determined by the flow cytometry (using anti-mouse IgG FITC) after a chase at 37ºC (internalization). The initial warming conditions to 37ºC were carefully controlled to ensure rapid activation of the endocytic machinery. Dots represent replicates of data obtained in independent experiments (n=8), and bars represent mean values. Kinetic parameters (k_{0-5} to k_{30-240}) were determined by calculation of a slope based on first-order kinetics process, and the kinetic segments are shown by the full line. Single exponential decay based on k_{0-5} is shown by the dotted line and double exponential decay based on k_{10-30} by the dashed line. R^2, the coefficient of determination for fitted curve. The mean R^2 value of individual experiments relative to the model was 0.963±0.019 (n=8). (**B**) *Confocal B/W images of cells representing the intracellular accumulation of mAb:eL^d after internalization*. Original images are presented in Fig. S2. Cell surface eL^d were labeled with 64-3-7 mAbs at 4ºC for 60 min and incubated at 37ºC for indicated periods of time (internalization). Remaining mAbs bound to the cell surface eL^d were acid stripped, and cells were fixed, permeabilized, and stained with AF^{555}-anti-mouse IgG$_{2b}$. Bars, 10 µm.

2. Open L^d conformers are sorted into late endosomes

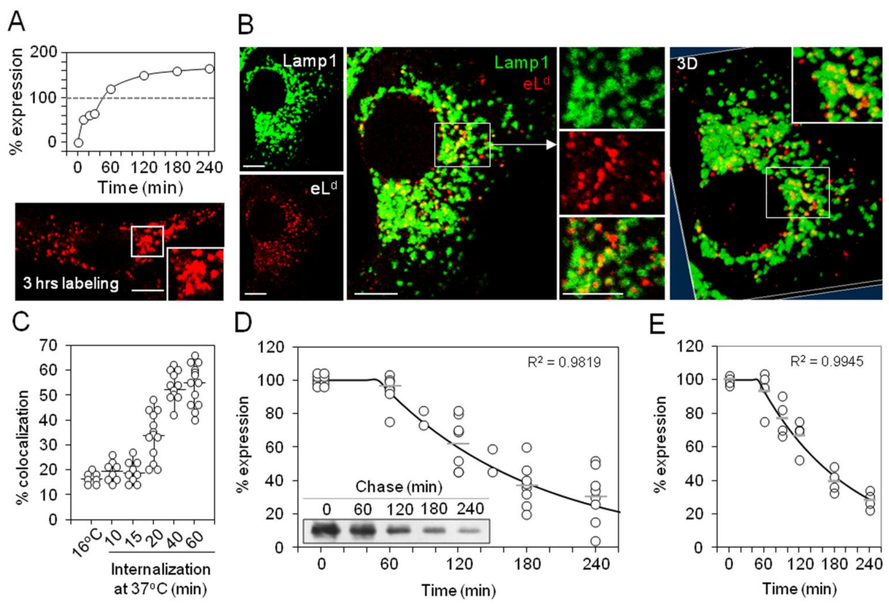

Figure 2. **Analysis of late trafficking events of empty L^d molecules.**
(**A**) MAb:eL^d *are retained in endosomes*. Cells were cultivated in the medium containing mAb 64-3-7 for indicated times, and cell surface and intracellular bound mAbs quantified by FITC-anti-mouse IgG using flow cytometry. Data represent intracellular expression relative to the cell surface of the same cell. Shown is a representative experiment (n=4). Confocal image of the representative cell after 3 hrs exposure to mAb is shown at the bottom. (**B**) *mAb:eL^d distribute into LEs*. Representative confocal images of the steady-state Lamp1 and mAb:eL^d after 45 min of internalization from the cell surface. Internalized mAb:eL^d were visualized using AF^{555}-anti-mouse IgG$_{2b}$ and cells simultaneously stained for Lamp1 using rat-anti-Lamp1 mAb and AF^{488}-anti-rat IgG. Images from the boxed area were acquired at higher magnification (z = 0.2 µm). The same cell is shown in 3D of stacked images. Bars, 10 µm. (**C**) *Early colocalization of mAb:eL^d with Lamp1*. Colocalization of eL^d with Lamp1 after various periods of internalization at 37ºC. The threshold level was established by 60 min internalization at 16ºC. Dots represent measurements on an individual cell, and horizontal bars mean values. Raw data are presented in Fig. S4 (**D**) *Internalized eL^d reach lysosome within 55 min*. Cell surface eL^d were biotinylated at 4ºC, the cells were chased at 37˚C, cellular eL^d immunoprecipitated from NP40 extracts and eL^d bands quantified after electrophoresis and chemiluminescence imaging (insert is an example). The degradation rate constant was calculated by single exponential first-order rate reaction. R^2, the coefficient of determination for fitted curve. The mean R^2 value of individual experiments relative to the model was 0.927±0.036 (n=8). (**E**) *Binding of mAbs to eL^d does not change the rate of degradation*. Degradation of mAb:eL^d after periods of internalization was determined by flow cytometry on permeabilized cells and the rate constant determined by the best fit as single exponential first-order rate reaction. Dots represent measurements in independent experiments, and horizontal bars mean values. The mean R^2 value of individual experiments relative to the model was 0.902±0.060 (n=4).

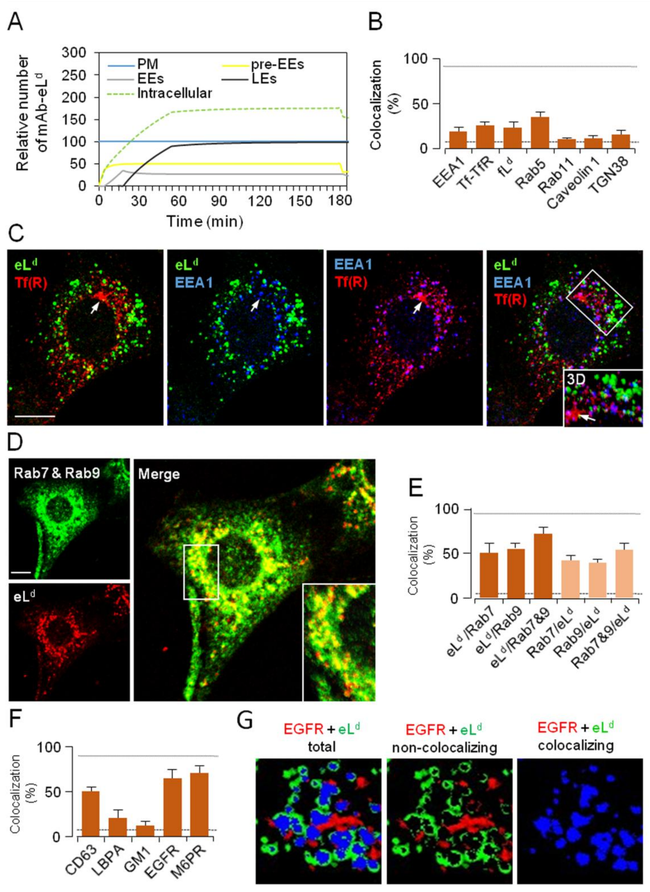

Figure 3. **Enrichment of mAb:eL^d in late endosomes by continous exposure to mAbs.**
(A) *Simulation of PM and endosomal distribution of mAb:eL^d under conditions of continuous presence of mAb 64-3-7*. The parameters from Fig. 4B were used, and calculations were described in the Supplementary Materials and Methods. The steady-state amount of mAb:eL^d at the PM was maintained constant (100%), and 25% of LE eL^d was set up to distribute into recycling membranes of LEs. **(B)** Colocalization of mAb:eL^d after 3 hrs of labeling with mAb 64-3-7 with markers of the EE system (EEA1, Rab5, Rab11, and caveolin 1), EE cargo proteins (internalized TfR and fL^d), and trans-Golgi network (TGN-38). Data represent mean±S.D. per cell, measured in three independent experiments (n=15-25 cells). Colocalization of EEA1 with GM_1 (dashed line) and K^d with D^d (dotted line) was used as the threshold level for minimal and maximal expected colocalization, respectively. **(C)** Confocal images of the three hrs-labeled eL^d (green) with TfR (1 hr labeling with AF^{555}-transferrin, red) and the steady-state EEA1 (blue). The boxed area is shown in the 3D presentation of stacked images. Bar, 10 µm. **(D)** Confocal images of mAb:eL^d after 3 hrs of labeling with mAb 64-3-7 and the steady-state Rab7+Rab9. The complete experiment is presented in Fig. S8. The boxed area is acquired at higher magnification. Bar, 10 µm. **(E)** Colocalization analysis of Rab7 and/or Rab9 with the three hrs labeled eL^d based on M1/M2 coefficients of pixel overlap. Data represent mean±S.D. per cell, measured in three independent experiments (n=18). **(F)** Colocalization of the three hrs-labeled eL^d with LE markers (CD63, LBPA, and GM1) and cargo molecules (EGFR and M6PR) that populate luminal membranes of degradative and non-degradative MVBs. Representative images are presented in Fig. S9. (**G**) Colocalization of perinuclear mAb:eL^d-loaded LEs (3 hrs labeling with 64-3-7) that are reached by internalized AF^{555}-EGF-labeled EGFRs (30 min internalization). Colocalizing areas were shown in blue.

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II. RECYCLING OF OPEN L^d CONFORMERS THROUGH LATE ENDOSOMES

1. Simulation of endosomal trafficking of eL^d

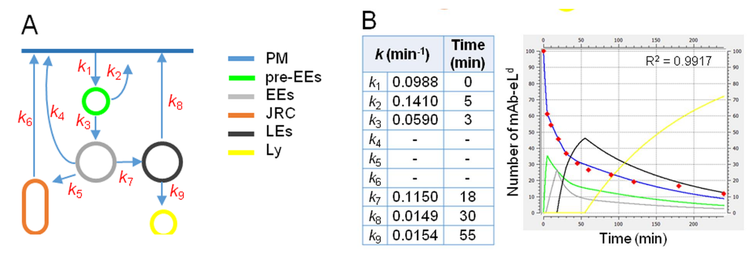


Figure 4. **Simulation of endosomal trafficking of eL^d.**
(A) *Schematic diagram of kinetic parameters used for simulation*. Distribution of mAb:eL^d at the plasma membrane (PM) and endosomal compartments was simulated using a multicompartment model based on first-order rate trafficking kinetic. Each, the earliest endosomal subsets (pre-EE), early endosomes (EE), juxtanuclear recycling compartment (JRC) and late endosomes (LE) were considered as one compartment, irrespective to their complex composition. Lysosomes (Ly) were considered as a separate compartment, and lysosomal distribution represents degradation. Rate constants (k_1-k_9) and the time of the onset of transition (time) were estimated by the best-fit optimization to the experimental data. **(B)** *Estimation of kinetic parameters that fit the experimental data provided in Fig. 1* (red diamonds). Tables contain kinetic parameters introduced into simulation (rate constants and time of onset). Image represent the screenshot of the software used for modeling. R^2, the coefficient of determination.

2. Open L^d conformers are recycled to the cell surface

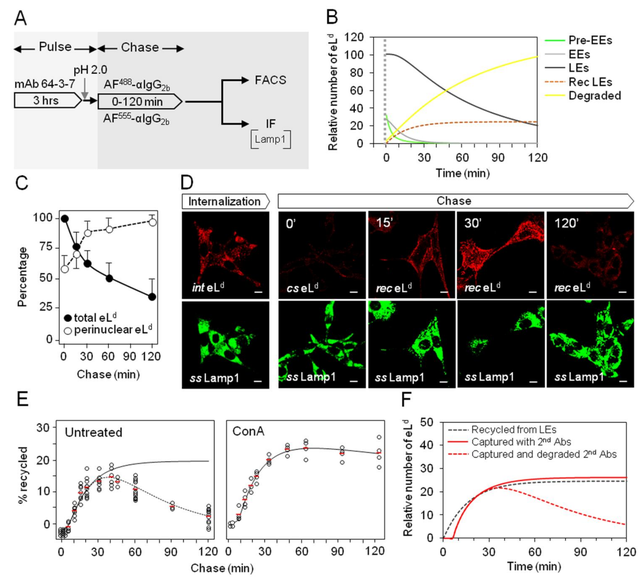


Figure 5. **Recycling of late endosomal eL^d.**
((**A**). *Experimental design*. Cells were exposed to mAb 64-3-7 for 3 hrs (pulse), cell surface-bound mAbs acid stripped (pH 2.5, 2 min), cells incubated 3 min in fresh medium followed by incubation (chase) up to two hours in medium with 2ndAbs, either FITC-anti-mouse IgG or AF^{555}-anti-mouse IgG$_{2b}$ to capture recycled mAb:eL^d. After the chase, the fluorescence signal was quantified by flow cytometry (FACS) or cells were fixed, permeabilized and stained to visualize Lamp1 (AF^{488}-anti-rat IgG). (**B**) *Simulation of the chase conditions*. MAb:eL^d distribution in endosomal compartments, recycling from LEs, and degradation during the chase was modeled using kinetic parameters from Fig. 4B. (**C**) *Intracellular dynamics of mAb:eL^d during the chase*. Quantitative analysis of the intracellular fluorescence of mAb:eL^d in the whole cell (total) and the perinuclear area (confined by Lamp1 expression) after indicated periods of the chase. Data represent mean ± S.D. per cell, measured in three independent experiments (n=19). (**D**) *Visualization of 2ndAb captured mAb:eL^d*. Images of intracellular distribution of mAb:eL^d after 3 hrs of labeling (*int* eL^d), at the cell surface after acid stripping (*cs* eL^d), and both cell surface and intracellular after chasing in the presence of fluorescent 2ndAbs (*rec* eL^d). The same cells were permeabilized and stained to show Lamp1 expression. Bars, 10 µm. (**E**) *Quantification of recycling from LEs*. Flow cytometric quantification of mAb:eL^d recycling in untreated cells and cells treated with Concanamycin A (ConA, 100 nM) during the chase. Dots represent replicates of data obtained in independent experiments, and bars represent mean values. Kinetic modeling of recycling in the presence (dashed line) and absence (full line) of degradation of reinternalized captured complexes. R^2, the coefficient of determination for fitted curve. The mean R^2 value of individual experiments for untreated and ConA-treated cells relative to the model (dotted and full line, respectively) was 0.777±0.140 (n=13) and 0.912±0.04 (n=4), respectively. (**F**) *Simulation of 2ndAb capture*. Simulation of recycling from LEs (dashed gray line) during conditions presented in Fig. 6B and kinetics of presentation of captured 2ndAb:mAb:eL^d complexes in the absence (full red line) and the presence (dashed red line) of their degradation. The onset of 2ndAb:mAb:eL^d degradation was set to 20 min and the rate constant of degradation to 0.014 min^{-1}, as determined in an independent experiment.

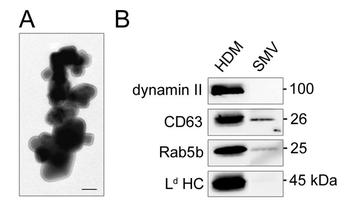


Figure 6. **Analysis of eL^d loading in secreted membrane vesicles.**
(**A**) Electron micrograph of isolated secreted membrane vesicles. Bar, 100 nm. (**B**) Western-blot analysis of high-density membranes (HDM) and secreted membrane vesicles (SMV).

3. The role of Rab7 and cholesterol unloading in degradation and recycling of open L^d conformers

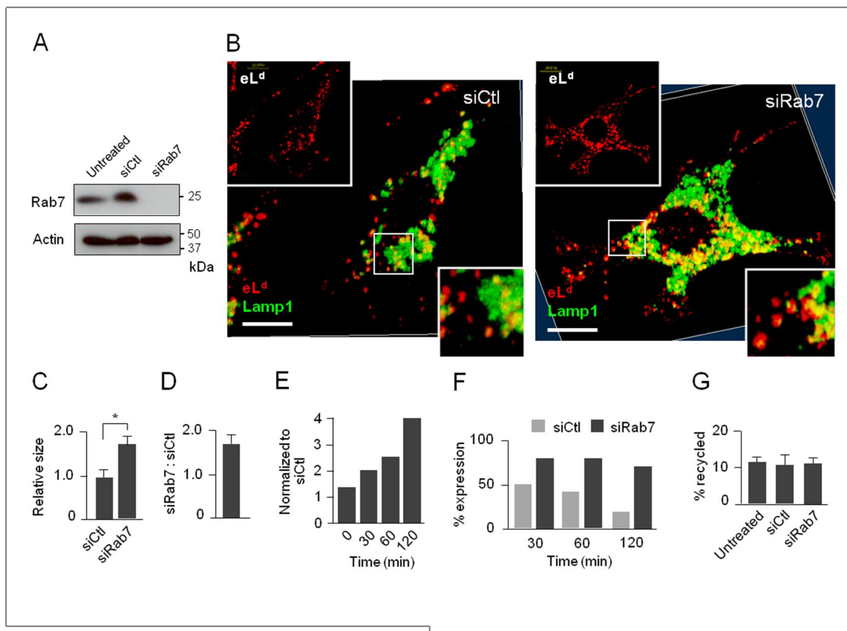
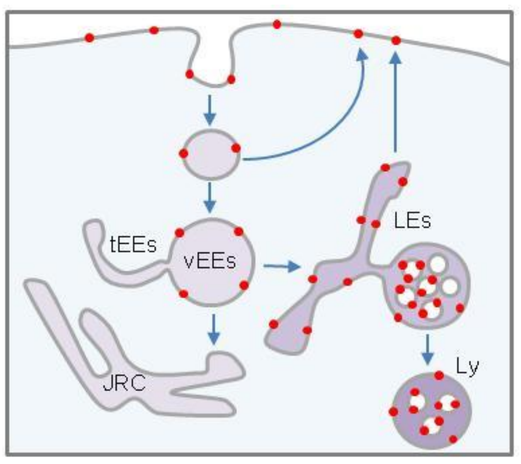


Figure 7. **Effect of Rab7 silencing on the LE route of eL^d.**
(**A**) Immunoblots of Rab7 and actin from Balb 3T3 cells 72 hours after transfection with scrambled RNA (siCtl) and Rab7 siRNA (siRab7). (**B**) 3D reconstruction of stacked images of the three hrs-labeled eL^d and the steady-state Lamp1 in transfected cells (72 hrs post transfection). Upper inserts present a confocal image of a single section and lower inserts enlargement of the boxed area. Bars, 10 µm. (**C**) The size of eL^d-loaded carriers in siCtl- and siRab7-transfected cells relative to the size of untransfected cells, determined on confocal images by the ImageJ plugin. Results are means±S.D. of three independent experiments (n=18 cells per experiment). (**D**) The ratio of intracellular eL^d in siRab7 and siCtl transfected cells after the three hrs labeling determined by flow-cytometry in permeabilized cells. Results are means±S.D. of three independent experiments. (**E**) Quantification of eL^d immunofluorescence signal by flow cytometry in transfected cells after 3 hrs of pulse labeling with mAbs 64-3-7 and 30, 60 and 120 min of the chase in the absence mAbs. Data represent the relative fluorescence signal normalized to siCtl-transfected cells. Shown is the representative experiment (n=3). (**F**) Kinetics of degradation of mAb:eL^d in transfected cells determined by quantification of confocal images. Shown is the representative experiment (n=3). (**G**) Recycling of the three hrs-labeled eL^d in transfected cells, determined by the flow cytometric assay. Results are means±S.D. of three independent experiments.

Conclusion:



Empty L^d molecules recycle through late endosomal compartments. They do not reqiure Rab7 membrane domains, but require vectorial LE motility, suggesting that LE recycling occurs from dynamic tubulovesicular LE domains prior segregation of eL^d in MVBs.

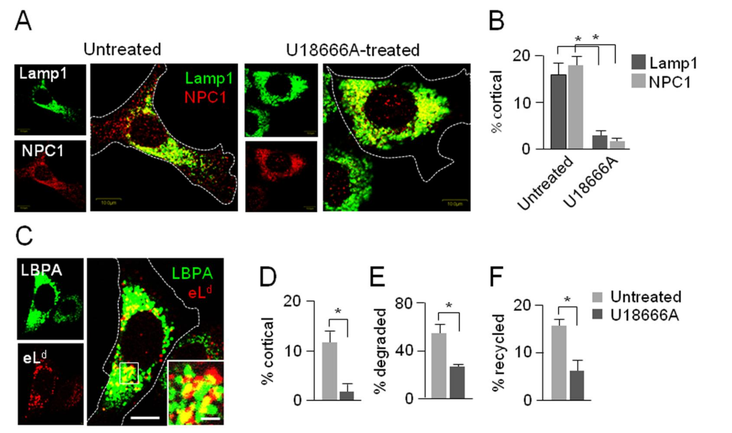


Figure 8. **LE trafficking of internalized eL^d in cells with reduced LE motility.**
(**A**) Confocal images of Lamp1 and NPC1 distribution in untreated and U18666A-treated (24 hrs, 6 µg/ml) cells. Bars, 10 µm. (**B**) Relative distribution of Lamp1 and NPC1 outside the perinuclear area in untreated and U18666A-teated cells (24 hrs). Data represent the percentage of fluorescence intensity per cell outside the perinuclear area. Results are means±S.D. of three independent experiments (n=15-20 cells per experiment). (**C**) Confocal images of the three hrs-labeled eL^d and steady-state LBPA in cells treated for 24 hrs with U18666A. Insert presents an enlargement of the boxed area. Bars, 10 µm. (**D**) Percentage of eL^d outside the perinuclear area measured by quantification of stacked double-stained (eL^d and Lamp1) confocal images. The perinuclear area is confined to the edges of continuous Lamp1-positive membranes. Results are means±S.D. of three independent experiments (n=15-20 cells per experiment). (**E**) Percentage of degraded mAb:eL^d in untreated and U18666A-teated (24 hrs) cells measured by the flow cytometric assay. Results are means±S.D. of three independent experiments. (**F**) Percentage of the recycled LE mAb:eL^d after 30 min of recycling determined by the flow cytometric assay. Results are means±S.D. of three independent experiments.

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