Plasticity of Polyubiquitin Recognition as Lysosomal Targeting Signals by the Endosomal Sorting Machinery[□]

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Lysosomal targeting is fundamental for the regulated disposal of ubiquitinated membrane proteins from the cell surface. To elucidate ubiquitin (Ub) configurations that are necessary and sufficient as multivesicular body (MVB)/lysosomal-sorting motifs, the intraendosomal destination and transport kinetics of model transmembrane cargo molecules bearing monoubiquitinated, multi-monoubiquitinated, or polyubiquitinated cytoplasmic tails were determined. Monomeric CD4 chimeras with K63-linked poly-Ub chains and tetrameric CD4-mono-Ub chimeras were rapidly targeted to the lysosome. In contrast, lysosomal delivery of CD4 chimeras exposing K48-linked Ub chains was delayed, whereas delivery of monoubiquitinated CD4 chimeras was undetectable. Similar difference was observed in the lysosomal targeting of mono- versus polyubiquitinated invariant chain and CD4 ubiquitinated by the MARCH (membrane-associated RING-CH) IV Ub ligase. Consistent with this, Hrs (hepatocyte growth factor regulated tyrosine kinase phosphorylated substrate), an endosomal sorting adaptor, binds preferentially to K63-Ub chain and negligibly to mono-Ub. These results highlight the plasticity of Ub as a sorting signal and its recognition by the endosomal sorting machinery, and together with previous data, suggest a regulatory role for assembly and disassembly of Ub chains of specific topology in lysosomal cargo sorting.

INTRODUCTION

Ubiquitination, a reversible posttranslational modification, serves as a sorting signal at multiple cellular locations in yeast and mammalian cells, including the *trans*-Golgi network (TGN), plasma membrane and early endosomes (Katzmann *et al.*, 2002; Bonifacino and Traub, 2003; Hicke and Dunn, 2003). Although cell surface resident polypeptides can be transiently down-regulated by internalization, regulated degradation of receptors and transporters requires postendocytic delivery to lysosomes, a process that can be signaled by ubiquitination (Di Fiore *et al.*, 2003; Dupre *et al.*, 2004).

Conjugation of a single Ub (herein after designated mono-Ub) was proposed to be sufficient as an endocytic and vacuolar sorting signal in yeast and mammalian cells (Hicke, 2001; Mosesson *et al.*, 2003). Recent evidence, however, suggests that multiple mono-Ub (designated multimeric-Ub) or Ub chains (designated polymeric-Ub or poly-Ub) are associated with efficient endocytic cargo recognition in animal cells (e.g., epidermal growth factor receptor [EGFR], major histocompatibility complex [MHC] I, and MHC II; Duncan *et al.*, 2006; Huang *et al.*, 2006; Ohmura-Hoshino *et al.*, 2006; Shin *et al.*, 2006; van Niel *et al.*, 2006), as well as in yeast (e.g., Gap1 amino acid permease, Fur4p uracil permease; Springael *et al.*, 1999; Dupre and Haguenaier-Tsapais, 2001; Soetens *et al.*, 2001; Dupre *et al.*, 2004).

After internalization, ubiquitinated cargo is recognized by the Ub-interacting motif (UIM) of hepatocyte growth factor-regulated tyrosine kinase phosphorylated substrate (Hrs) and STAM (Williams and Urbe, 2007), mammalian orthologues of the yeast Vps27 and Hse1, respectively (Hurley and Emr, 2006). These are conserved multidomain adaptors of ESCRT 0 (the endosomal sorting complex required for transport; Hurley and Emr, 2006; Williams and Urbe, 2007). Besides recognizing ubiquitinated cargo, the Hrs/STAM complex binds to PtdIns3P and clathrin heavy chain, facilitating clathrin recruitment to the limiting membrane of early endosomes, a major component of the endosomal double-layered coat (Raiborg *et al.*, 2002). Recognition and concentration of ubiquitinated cargo by the Hrs/STAM complex at the double-layered coat is a prerequisite for cargo segregation and targeting to lysosomes (Raiborg *et al.*, 2002). Successive cargo transfer from ESCRT0 to ESCRT I, II, and III coincides with endosomal maturation, manifesting in compositional changes, luminal acidification, and inward vesicular budding that leads to the formation of late endosomes enriched in multivesicular bodies (MVB; Mellman, 1996; Mukherjee *et al.*, 1997). MVB formation ensures that cytosolic domains of transmembrane proteins become accessible to lysosomal proteases, a process promoted by endo-lysosomal fusions (Gruenberg and Stenmark, 2004; Hurley and Emr, 2006).

The essential role of poly-Ub or multimeric-Ub in rapid internalization of transmembrane proteins (e.g., CD4, β_2 -adrenergic receptor complex, MHC I, MHC II, and ENaC; Traub and Lukacs, 2007) suggested that polyvalent interactions are required to overcome the low-affinity binding of Ub to the UIM of Ub-binding clathrin adaptors (e.g., epsin and eps15; Barriere *et al.*, 2006; Hawryluk *et al.*, 2006). Elucidating the significance of Ub configuration in lysosomal sorting is hampered by difficulties to discriminate between

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the role of Ub as an endocytic and lysosomal-sorting signal. Although previous observations suggest that short poly-Ub chains with various topologies may mediate lysosomal targeting of plasma membrane proteins (Staub *et al.*, 1997; Geetha *et al.*, 2005; Duncan *et al.*, 2006; Kamsteeg *et al.*, 2006; Wiemuth *et al.*, 2007), the Ub configuration that is necessary and sufficient to signal lysosomal sorting has not been determined. Because the UIMs of epsin and eps15/eps15R, Ub-binding clathrin adaptors, as well as of Hrs/STAM appear to be critical for ubiquitinated cargo recognition, we hypothesized that similar principles may prevail for Ub recognition at the cell surface and endosomes.

To address the Ub configuration requirement for endolysosomal sorting, we used chimeric models and CD4, exposing mono-Ub, multimeric-Ub, or poly-Ub in their cytoplasmic tails. Interorganellar transport kinetics of cargoes with different Ub configurations were determined by vesicular pH measurements using fluorescence ratio image analysis (FRIA) of fluorophore-labeled cargoes. The results show that both multimeric and polymeric, but not monomeric Ub are efficiently recognized as lysosomal-targeting signal by the endosomal sorting machinery, consistent with the Ub-binding preference of Hrs.

MATERIALS AND METHODS

Cells Lines

COS-7, HEK293T, E36, and ts20 cells were grown in Dulbecco's modified Eagle's medium (DMEM) as described (Barriere *et al.*, 2006). HEK293T cells were stably transfected with the pIRES2-puro expression construct (BD Biosciences, San Jose, CA) and CD4TI- or CD4TIUb-expressing clones were selected in the presence 10 μ g/ml puromycin.

Transient transfection of COS-7, HEK293, and ts20/E36 cells was performed as described (Barriere *et al.*, 2006). For transient cotransfection, the Ub variant and cargo cDNA was used at a 3:1 mass ratio.

DNA Constructs and Antibodies

CD4-Ub chimeras with the Lys-less linker (TL) or the tetramerization coiled-coil domain (cc) from the potassium channel Kir6.2 have been described (Zerangue *et al.*, 1999; Barriere *et al.*, 2006). The terminal glycine residues in Ub were deleted by PCR mutagenesis (CD4TI-UbR Δ G and CD4TI-Ub Δ G) using the wild-type (wt) Ub and the Lys-less Ub (UbR) cDNA as template (provided by Dr. L. Hicke, Northwestern University, Evanston, IL). Lys residue was inserted into the UbR Δ G moiety of CD4TI-UbR Δ G at the 6, 29, 48, or 63 position by overlapping PCR. In CD4-1K, the CD4 cytoplasmic tail (MS-QIKRLLEKKTQCQPHRFQKTCSPF) was substituted with RMSQIRRAASER-KTCQCPHRFQ peptide to replace Lys436 and Lys442 residues with Arg and the di-Leu endocytosis motif with Ala, indicated by underscored letters. Thus the CD4-1K cytoplasmic tail contains a single Lys (at position 443) and lacks the last six-amino acid residues. All constructs were verified by DNA sequencing. HA-tagged Ub, UbR, UbR63K, and UbR48K (gift of Y. Yarden, Weizman Institute, Rehovot, Israel) have been described (Mosesson *et al.*, 2003).

Glutathione S-transferase (GST) fusions, containing one (GST-Ub), two (GST-2Ub), and three Ubs (GST-3Ub) were described (Barriere *et al.*, 2006). GST-Ub-L-Ub with a 10-amino acid linker between the Ubs and GST-Ub-Ub1A, containing Ub144A, were constructed by PCR mutagenesis. The GST-4Ub was generated by in frame insertion of PCR amplified Ub cDNA into the GST-3Ub. MBP-Hrs-His₆ and MBP-HrsS270A-His₆ were obtained by subcloning the mouse Hrs cDNA from pGEM-myc-Hrs and pGEM-myc-HrsS270A (provided by Dr. H. Stenmark, Norwegian Radium Hospital, Oslo, Norway) into pMAL-c2. The C-terminal His₆ was introduced by PCR.

The following antibodies were used: anti-CD4: RPA-T4 (BD Biosciences, Oakville, ON, Canada), OKT4 (Harlan Bioproducts, Indianapolis, IN), rat monoclonal (Serotec, Raleigh, NC) and rabbit polyclonal (H-370, Santa Cruz Biotechnology, Santa Cruz, CA). The Lamp1 (H4A3) mAb has been developed by J. August (University of Iowa) and J. Hildreth (University of Iowa) and was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). The rabbit anti-Hrs was kindly provided by Dr. H. Stenmark. The anti-Ub is a monoclonal anti-Ub horseradish peroxidase (HRP)-conjugated (P4D1, sc-8017 Santa Cruz).

Vesicular pH Measurement

To monitor the endocytic trafficking of internalized chimeras, CD4, and other transmembrane proteins, the vesicular pH (pH_v) of cargo-containing vesicles was determined by FRIA essentially as described for CFTR (cystic fibrosis

transmembrane conductance regulator; Sharma *et al.*, 2004). Cell surface CD4 (OKT4) and Lamp1 (Developmental Studies Hybridoma Bank) were labeled with the relevant primary Ab and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary Fab (Jackson ImmunoResearch Laboratories, West Grove, PA) by incubating primary (1/100) and secondary (1/100–500) Abs together routinely for 1 h at 37°C. Then cells were washed (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 10 mM glucose, 0.1 mM CaCl₂, and 1 mM MgCl₂, pH 7.3) and chased for 30–90 min at 37°C. Fluid phase Ab uptake was not detectable by FRIA in mock-transfected cells (data not shown).

Lysosomes were labeled overnight in the presence of FITC-dextran or Oregon488-dextran (50 μ g/ml, MW, 10 kDa, Molecular Probes, Eugene, OR) and chased >3 h (Poet *et al.*, 2006) or loaded for 15 min with 5 mg/ml FITC-dextran and chased for 90 min. Recycling endosomes were labeled with FITC-transferrin (Tf; 5 μ g/ml, 1 h labeling after 45-min serum depletion at 37°C) and chased for 10 min. FITC-Fab antibody, FITC-Tf, FITC-dextran, and FITC/Oregon488-loaded endolysosomes were imaged on an Axiovert 100 inverted fluorescence microscope (Carl Zeiss MicroImaging, Toronto, ON, Canada) at 35°C, equipped with a Hamamatsu ORCA-ER 1394 (Hamamatsu, Japan) cooled CCD camera and a Planachromat (63 $\times$, NA 1.4) objective. Fluorescence ratio image acquisition and analysis were performed with the MetaFluor software (Molecular Devices, Downingtown, PA). Images were acquired at 490 $\pm$ 5- and 440 $\pm$ 10-nm excitation wavelengths, using a 535 $\pm$ 25-nm emission filter.

Calibration curves, describing the relationship between the fluorescence ratio values and pH_v, served to calculate the luminal pH of individual vesicles after fluorescence background subtraction at both excitation wavelengths. In situ calibration was performed by clamping the pH_v between 4.5 and 7.4 in K⁺-rich medium (135 mM KCl, 10 mM NaCl, 20 mM HEPES or 20 mM MES, 1 mM MgCl₂, and 0.1 mM CaCl₂) in the presence of 10 μ M nigericin and 10 μ M monensin (Sigma-Aldrich, Oakville, ON, Canada) and recording the fluorescence ratio. In each experiment the pH_v of 200–800 vesicles was determined, precluding those located at the subplasma-membrane region. To ensure that comparable number of vesicles was evaluated for each construct, three times more cells were analyzed for chimeras with high recycling efficiency (e.g., CD4TI and CD4TI-UbR Δ G). As an internal control, one point calibration was performed on each coverslip by clamping the pH_v to 6.5 with monensin and nigericin. Mono- or multipeak Gaussian distributions of pH_v values were obtained with Origin 7.0 software (OriginLab, Northampton, MA). The mean pH_v of each vesicle population was calculated because the arithmetic mean of the data and was identical to the Gaussian mean, based on single-peak distribution fitting. At least three independent experiments were performed for each condition. HEK293T cells constitutively expressing CD4TI-Ub were overtransfected with Ub variants and the expression plasmid encoding the dsRed fluorescent proteins (BD Biosciences) at 10:1 ratio. FRIA was performed on cells expressing the dsRed only. The mean pH_v obtained in three or more experiments is shown in Supplementary Table S1.

To confirm that the primary and secondary Ab remains bound to CD4 in late endosomes, the pH resistance of Ab binding was measured. After primary and HRP-conjugated secondary Ab binding to CD4TI-expressing HEK293 cell at 0°C the pH of the extracellular medium was changed to pH 7.2, 5.0, and 2.5 for 5 min. HRP-conjugated Ab binding was measured by fluorescence, using Amplex-Red as substrate (data not shown). The Ab binding was virtually unaltered at pH 5.0, but was reduced by 95% at pH 2.5.

Considering that the pH sensitivity of FITC is reduced at pH <5 (pK_a ~6.4), we validated our pH_v measurements at pH_v <5. To this end the lysosomal compartment was loaded with a mixture of Oregon-Green (75%) and FITC-dextran (25%) to increase the detection sensitivity at acidic pH, because the Oregon-Green pK_a is ~4.7. FRIA showed that Oregon-Green/FITC-dextran loading provided pH values similar to that obtaining in the presence of FITC alone, verifying the extrapolation procedure for pH_v <5 based on FITC loading alone (data not shown). All experiments were performed in COS-7, and critical ones were repeated in HEK293 cells with similar results.

Immunofluorescence Microscopy

Lysosomes were labeled with anti-Lamp1 Ab or by FITC-dextran (50 μ g/ml, MW, 10 kDa), with loading as described for pH_v determination. Cells expressing the chimera were allowed to internalize CD4 antibody (RPA-T4, BD Biosciences) complexed with FITC-conjugated goat anti-mouse Fab or TRITC-conjugated goat anti-mouse IgG for 0.75–1 h in DMEM and chased for 0.5 h. During the last 45 min, cells were labeled with FITC-Tf or TRITC-Tf (5 μ g/ml) after 45-min serum depletion to visualize recycling endosomes. Single optical sections were collected by Zeiss LSM510 laser confocal fluorescence microscope, equipped with a Plan-Apochromat 63 $\times$ /1.4 (Carl Zeiss MicroImaging) as described (Lechardeur *et al.*, 2004). Images were processed with Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Recombinant Protein Purification and Pulldown Assay

GST-Ub fusions proteins were purified essentially as described (Barriere *et al.*, 2006). Expression of His₆-tagged MBP-HrsS270A, MBP-Hrs298X, and MBP was induced in HB101 cells with 0.3 mM IPTG (4 h, RT). MBP-Hrs-His₆ was expressed in the BL21(DE3) strain. For the Hrs pulldown experiments, the bacterial expression level of recombinant MBP-Hrs proteins was determined by affinity

purification using amylose beads. Next, ~65 pmol of fusion proteins was bound to amylose beads (overnight, 4°C), washed three times (20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM EDTA), and incubated with mono-Ub (15 µg, Sigma), K48-linked (15 µg), or K63-linked (6 µg) poly-Ub chain (Boston Biochem, Cambridge, MA) for 2 h at 4°C. Bound proteins were eluted with 2× LSB. Six percent of K63- and 16% of K48-linked poly-Ub and mono-Ub present in the binding buffer were loaded directly to compensate for the approximately twofold lower detection efficiency of K48 poly-Ub by the P4D1 anti-Ub Ab.

Cell Surface Density, Internalization, Recycling, and Stability of CD4-Ub Chimeras

The cell surface density and internalization of CD4-Ub chimeras was measured essentially as described previously (Barriere *et al.*, 2006), using anti-CD4 Ab. The remaining anti-CD4 Ab was detected by HRP-conjugated goat anti-mouse Ab and AmplexRed as a fluorescent substrate (Molecular Probes). Fluorescence was measured by OPTIMAstar (BMG Labtech, Offenbourg, Germany) plate reader.

Recycling of chimeras was monitored by the biotin-streptavidin sandwich techniques as described (Sharma *et al.*, 2004) with the modification that endosomes were loaded for 30 min with anti-CD4 and biotinylated anti-mouse Abs, and then the cell surface-resident biotinylated secondary Ab was blocked with 20 µg/ml streptavidin (Sigma-Aldrich; 4°C, 1 h). Exocytosis of internalized cargo was stimulated by shifting the temperature to 37°C for 5–10 min and detected by HRP-conjugated streptavidin and AmplexRed. Recycling efficiency was expressed as % of endocytosed cargo, measured in parallel samples.

The turnover of plasma membrane-associated CD4 chimeras was monitored by the disappearance kinetics of cell surface-bound anti-CD4 Ab during 0.5–20-h chase at 37°C in transiently transfected COS-7 cells. The cell surface bound anti-CD4 Ab was determined HRP-conjugated secondary Ab, using Amplex-Red as substrate.

Immunoprecipitation and Western Blotting

To determine the endogenous ubiquitination level of various CD4-chimeras at the cell surface and endosomes, the chimeras were labeled with anti-CD4 (OKT4) Ab for 30 min at 37°C in transiently transfected COS-7 cells. To delay degradation of the ubiquitinated chimeras the medium was supplemented with 20 µg/ml leupeptin, 20 µg/ml pepstatin, and 1 µM bafilomycin A1. Cells were lysed with RIPA buffer containing 10 µg/ml leupeptin and pepstatin, 100 µM phenylmethylsulfonyl fluoride, 10 µM MG132, and 10 mM NEM. Anti-CD4 Ab complexes were isolated on protein G agarose (Invitrogen, Carlsbad, CA; 2 h, 4°C), bound proteins were eluted with 2× LSB and probed with polyclonal anti-CD4 (H-370, Santa Cruz Biotechnology) or monoclonal anti-Ub HRP-conjugated (P4D1, sc-8017; Santa Cruz Biotechnology). To monitor the incorporation of heterologous Ub, CD4TI-Ub-expressing HEK293 cells were transiently transfected with HA-tagged Ubwt, UbR, UbR63K, or UbR48K. After 48 h, cells were lysed in RIPA buffer as above. CD4TI-Ub was precipitated with anti-CD4 rat monoclonal Ab (Serotec) and protein G agarose beads (Invitrogen). Bound proteins were probed with polyclonal anti-CD4 (H-370; Santa Cruz Biotechnology) or anti-HA Ab (Covance; MMS-101R).

Densitometric analysis of immunoblots was performed with the NIH Image 1.62 software as described (Sharma *et al.*, 2004). Immunoprecipitations of Hrs with CD4TI-Ub was carried out as outlined previously for CFTR (Sharma *et al.*, 2004) with the following modifications. HEK293 cells expressing CD4TI-Ub were incubated with MG132 (10 µM, 1 h) and lysed 150 mM NaCl, 20 mM Tris-Cl, and 0.2% NP-40, pH 7.4, containing 10 µg/ml leupeptin and pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride, 10 µM MG132, and 10 mM NEM. The chimera was precipitated with the OKT4 Ab.

Statistical Analysis

Experiments were repeated at least three times or as indicated. Data are means ± SEM. Significance was calculated by using the two-tailed *p* value at

Figure 1. Expression and stability of CD4-Ub chimeras. (a) Schematic representations of CD4-Ub chimeras. The cytoplasmic tail of CD4 was replaced with a flexible linker (CD4TI) or a tetramerization coiled-coil domain (CD4cc) and the indicated Ub moiety as described in *Materials and Methods*. (b) Polyubiquitination of the CD4TI-Ub. COS-7 transiently transfected cells expressing the indicated CD4 chimeras were incubated with OKT4 anti-CD4 antibody for 30 min at 37°C. CD4-Ab complexes were immunoprecipitated on protein G beads. Immunoprecipitates were probed with HRP-conjugated P4D1 anti-Ub antibody. (c) Metabolic stability of the CD4 chimeras was measured after inhibiting translation with 100 µg/ml cycloheximide (CHX) in transiently transfected HEK293 cells. After the chase, equal amounts of cell lysates were immunoblotted with anti-CD4 Ab. Ubiquitination of CD4TI-Ub is indicated by an asterisk (*). (d) Degradation kinetics of CD4-Ub chimera were determined by densitometry of immunoblots illustrated in panel c. Data are means ± SEM; *n* = 3–4. (e) Inhibition of lysosomal proteolysis and cargo delivery stabilizes CD4TI-Ub and CD4cc-UbRAG. The stability of CD4-Ub chimeras was measured in the presence of bafilomycin A1 (1 µM), MG132 (20 µM), or leupeptin+pepstatin (5–5 µg/ml) as described in panel c after a 6-h CHX chase. (f) Densitometric analysis of CD4TI, CD4TI-Ub, and CD4cc-UbRAG immunoblots as shown in panel e. Lactacystin at 10 µM was used. Data are expressed as percentage of the initial amount of cargo. Means ± SEM; *n* = 3.

95% confidence level with unpaired *t* test, using the Prism software (Graph-Pad Software, San Diego, CA).

RESULTS

CD4-Ub Chimeras to Probe Ubiquitination as a Lysosomal Targeting Signal

CD4 has been extensively used as a model transmembrane cargo molecule to establish the requirement and sufficiency of sorting signals (Pelchen-Matthews *et al.*, 1989; Pitcher *et al.*, 1999). The truncated CD4, consisting of the extracellular and transmembrane domain of CD4 (CD4TI) is targeted to the plasma membrane by default and lacks any sorting signal (Bedinger *et al.*, 1988; Barriere *et al.*, 2006). We engineered three CD4-Ub chimeras, exposing either a poly-Ub chain (CD4TI-Ub), an unextendable mono-Ub (CD4TI-UbRΔG), or a tetrameric unextendable Ub (CD4cc-UbRΔG) as their cytoplasmic sorting signal to assess the structural basis of Ub-dependent lysosomal sorting of transmembrane cargo (Figure 1a).

In CD4TI-Ub, the wt Ub was fused to a truncated CD4 (CD4TI). To prevent Ub-chain formation, all lysine residues were replaced with arginines and the C-terminal terminal Gly residues were also deleted in the Ub moiety (CD4TI-UbRΔG, Figure 1, a and b). Deletions of Gly residues prevented the possible recruitment of the ubiquitination machinery (Reyes-Turcu *et al.*, 2006). Finally, to mimic multiple monoubiquitination (Haglund *et al.*, 2003), the cytoplasmic linker of CD4TI-UbRΔG was substituted with the cc tetramerization domain of the Kir6.2 channel (CD4cc-UbRΔG; Yuan *et al.*, 2003). This tetramerization domain provoked the oligomerization of soluble and chimeric proteins (Barriere *et al.*, 2006) and was postulated to expose tetrameric, unextendable Ub.

To demonstrate the differential ubiquitination of the three model proteins, the cell surface resident and internalized CD4-Ub chimeras were immunoprecipitated after anti-CD4 antibody binding and endocytosis, as described in *Materials and Methods*. This approach precluded the detection of ubiquitinated chimera associated with the endoplasmic reticulum (ER) and Golgi compartments. Probing the immunoprecipitates with anti-Ub showed that CD4TI-Ub is subjected to polyubiquitination. In contrast, we could not detect Ub conjugation to CD4TI-UbRΔG and CD4cc-UbRΔG (Figure 1b), supporting the prediction that these fusion proteins expose a single, covalently attached Ub in vivo.

Poly-Ub and Multimeric-Ub Metabolically Destabilizes CD4 Reporter Proteins

The metabolic stability of chimeras was measured by immunoblotting after the inhibition of protein synthesis with cycloheximide (CHX). Both CD4TI-Ub and CD4cc-UbRΔG have significantly faster turnover rates than either CD4TI or CD4TI-UbRΔG (Figure 1, c and d). The accelerated degradation of CD4TI-Ub and CD4cc-UbRΔG, at least in part, can be attributed to lysosomal proteolysis, because preventing lysosomal acidification and delivery by bafilomycin A1, a vacuolar H⁺-ATPase inhibitor, or blocking lysosomal cathepsins by leupeptin/pepstatin, significantly delayed the degradation (Figure 1, e and f; van Weert *et al.*, 1995; Aniento *et al.*, 1996). The cysteine protease and proteasomal inhibitors MG132 and lactacystin also stabilized the chimeras, conceivably by depleting endogenous Ub and/or interfering with cargo budding into MVB, as documented for ubiquitinated EGF and PDGF (platelet-derived growth factor) receptors (Longva *et al.*, 2002; Figure 1, e and f).

To substantiate the differential stability of CD4-Ub chimeras, their plasma membrane turnover was determined next.

Figure 2. Stability, internalization and recycling of CD4-Ub chimeras. (a) The turnover of plasma membrane pool of chimeras was monitored by the disappearance of cell surface bound anti-CD4 Ab in transiently transfected COS-7 cells. Cells were labeled with anti-CD4 Ab on ice for 1 h and then chased for 0.5–20 h at 37°C. The Ab remaining at the cell surface was measured by fluorescence in the presence of HRP-conjugated secondary Ab and AmplexRed, as described in *Materials and Methods*. The fluorescence was normalized for cellular proteins. Means $\pm$ SEM; *n* = 3. (b) Internalization rates of CD4-Ub chimeras. Endocytic rates of chimeras were measured by anti-CD4 Ab uptake in COS-7 cells as described in *Materials and Methods*. Data are expressed as percentage of the initial amount of CD4-Ub chimeras at the cell surface. Means $\pm$ SEM; *n* = 3–5. (c) Recycling efficiency of chimeras was measured in transiently transfected COS-7 cells with the biotin-streptavidin sandwich technique as described in *Materials and Methods*. Recycling was expressed as the percentage of internalized cargo. Means $\pm$ SEM; *n* = 3–5.

Chimeras confined to the plasma membrane were labeled with anti-CD4 Ab on ice and then chased for 0–20 h at 37°C. Anti-CD4 Ab remaining at the cell surface was measured by the fluorescence generated in the presence of HRP-conjugated anti-mouse Ab and Amplex-Red. Cell surface remaining Ab was expressed as the percentage of the initial amount (Figure 2a and see *Materials and Methods*). The cell surface turnover of the monoubiquitinated CD4TI-UbRΔG ($T_{1/2}$ ~9.8 h) similar to CD4TI ($T_{1/2}$ ~16 h), was several-fold slower than that of the CD4TI-Ub ($T_{1/2}$ ~25 min) or CD4cc-UbRΔG ($T_{1/2}$ ~34 min), representing poly- and multiubiquitinated cargo, respectively (Figure 2a).

The plasma membrane turnover of chimeras is determined by their internalization, degradation and recycling rates at constant biosynthesis (Supplementary Figure S1a). Accelerated internalization of multi- and polyubiquitinated chimeras likely contributes to their faster lysosomal degradation by increasing cargo concentration in sorting endosomes. We and others have demonstrated that the internalization rates of poly- and multiubiquitinated cargoes are significantly faster than their monoubiquitinated counterpart (Springael *et al.*, 1999; Barriere *et al.*, 2006; Hawryluk *et al.*, 2006). These differences were also confirmed by measuring initial rates of anti-CD4 Ab uptake by the chimeras (Figure 2b). Endocytosis of both the multimeric CD4cc-

Figure 3. Subcellular localization of internalized CD4-Ub chimeras. (a) Internalized CD4TI-Ub and CD4cc-UbRΔG colocalizes with lysosomes and avoid recycling endosomes in HEK293 cells. Chimeras were internalized with anti-CD4 Ab and FITC-conjugated secondary Fab. Lysosomes and recycling endosomes were labeled with FITC-dextran and TRITC-Tf, respectively. Internalized dextran was colocalized with Lamp1 by immunostaining (data not shown). Single optical sections were obtained by laser confocal fluorescence microscopy. Bar, 10 μ m. (b) Internalized CD4TI and CD4TI-UbRΔG colocalize with recycling endosomes and excluded from lysosomes. Staining of recycling endosomes, lysosomes, and CD4 chimeras were performed as described in panel a. Bar, 10 μ m.

UbRΔG and the polyubiquitinated CD4TI-Ub was 3–4-fold faster (~ 45 – 50% /5min) than CD4TI, CD4TI-UbRΔG, or CD4TI-UbR (Figure 2b; Barriere *et al.*, 2006). Importantly, the internalization of monoubiquitinated cargo (CD4TI-UbRΔG and CD4TI-UbR) was significant ($\sim 13\%$ /min and $\sim 8\%$ /min) and comparable to that of the CD4TI reporter molecule ($\sim 12\%$ /min) and thus conceivably was mediated by bulk flow endocytosis (Barriere *et al.*, 2006). The relatively high cell surface density of monoubiquitinated cargo despite its slower internalization rate ensured sufficient Ab labeling of endocytic vesicles for immunolocalization and pH measurements (see below).

Poly-Ub and Multimeric-Ub Impedes Recycling and Targets Reporter Molecules into Lysosomes

Besides accelerated internalization, endosomal retention and lysosomal sorting may account for the rapid cell surface turnover of multi- and poly-ubiquitinated model cargoes as proposed for misfolded CFTR and yeast H^+ -ATPase (Sharma *et al.*, 2004; Liu and Chang, 2006) and other ubiquitinated membrane proteins (Gruenberg and Stenmark, 2004; Traub and Lukacs, 2007). To address these possibilities, the recycling rates of chimeras were determined by the biotin-streptavidin sandwich technique as described in *Materials and Methods*. Although 34–38% of internalized CD4TI and monoubiquitinated CD4TI-UbRΔG returned to the cell surface in 5 min, the recycling efficiency of poly- and multiubiquitinated cargo was attenuated by two- to threefold (11–14%/5 min; Figure 2c). Intriguingly, preserving the terminal glycines in the CD4TI-UbR decreased the recycling efficiency by 50% as compared with CD4TI-UbRΔG (Figure 2, b and c), whereas it had no discernable effect on the internalization (Barriere *et al.*, 2006), suggesting that terminal glycines may contribute to lysosomal sorting. These results, jointly, indicate that both accelerated internalization and defective recycling contribute to the rapid cell surface turnover of the poly-Ub- and multimeric-Ub-containing model proteins.

The destination of endocytosed chimeras was established first by colocalization studies using organellar markers and laser confocal fluorescence microscopy in HEK293 and COS-7 cells. Chimeras were labeled with anti-CD4 Ab and fluoro-

phore-conjugated secondary Fab internalization and chased for 30 min in Ab-free medium at 37°C. CD4TI-Ub and CD4cc-UbRΔG was predominantly targeted to lysosomes, identified by the fluid-phase marker dextran (Figure 3) or Lamp1 immunostaining (data not shown) and largely was excluded from recycling endosomes, visualized by FITC-Tf (Figure 3a). Conversely, endocytosed CD4TI-UbRΔG carrying an unextensible Ub, similarly to CD4TI, was sorted to recycling endosomes and excluded from lysosomes (Figure 3b). Considering that none of the above experiments allowed us to delineate the significance of ubiquitin configuration as lysosomal-sorting signal due its compounding effect on endocytosis (see Figure 2b), we designed an assay to measure the transfer kinetics of chimeras from early endosomes to lysosomes. The assay monitors the pH_v and relies on the distinct pH of endolysosomal compartments.

Monitoring Ubiquitinated Cargo Sorting from Early Endosomes in Live Cells

The luminal pH of recycling endosomes is more alkaline ($pH \sim 6.4$ – 6.5) than sorting endosomes ($pH \sim 5.9$ – 6.3), whereas maturation of early endosome into MVB/late endosome and lysosome is accompanied by their acidification to $pH \sim 5.5$ – 6 and <5.5 , respectively (Mukherjee *et al.*, 1997; Supplementary Figure S1a). Therefore, cargo localization could be inferred from the pH of cargo-containing vesicles measured by fluorescence ratio image analysis (FRIA; see *Materials and Methods*), using cargo-bound FITC, a pH-sensitive fluorophore (Ohkuma and Poole, 1978).

To validate the assay, the destination of FITC-conjugated Tf, dextran, and EGF was established. The FRIA analysis confirmed that internalized Tf was confined to mildly acidic ($pH_v 6.35 \pm 0.08$, $n = 3$) recycling endosomes, whereas dextran and EGF were accumulated in acidic ($pH_v 4.60 \pm 0.08$ and 4.98 ± 0.15 , $n = 3$) lysosomes (Figure 4a). These pH_v values are similar to those reported for the respective organelles in fibroblast, BHK, and CHO cells (Mukherjee *et al.*, 1997). The mean pH_v value obtained in three or more independent experiments is indicated in the text and in Supplementary Table S1, whereas the result of a single experiment is depicted in figures.

Figure 4. Monitoring internalized cargo sorting by vesicular pH (pH_v) measurement. (a) Validation of pH_v measurements by monitoring transferrin (Tf), EGF (epidermal growth factor), and dextran sorting in HEK293 cells. The loading protocols of FITC-dextran and FITC-Tf are described in *Materials and Methods*. Serum-depleted cells internalized biotin-EGF and FITC-streptavidin for 1 h and chased for 2 h at 37°C. The pH_v was measured by FRIA. Similar results were obtained in COS-7 (dextran) cells. The mean ($\pm$ SEM) pH_v and the number of vesicles analyzed in a single experiment are indicated. The mean pH_v from ≥ 3 independent experiments are summarized in Supplementary Table S1. (b) The kinetic of CD4TI-Ub lysosomal delivery. Anti-CD4 antibody and FITC-conjugated secondary Fab was bound to transfected HEK293 cells for 1 h at 0°C. Then the temperature was raised to 37°C for 1, 15, or 30 min, and the pH_v was measured by FRIA. (c) CD4TI- and CD4TI-Ub-expressing HEK293 cells were allowed to internalize anti-CD4 primary Ab and FITC-conjugated secondary Fab for 1 h at 37°C and were chased for 30 min before FRIA. Similar results were obtained in COS-7 cells (data not shown).

To determine the sorting kinetics of cargo between early endosomes and lysosomes as well as other destinations, CD4TI and CD4TI-Ub were labeled with anti-CD4 Ab and secondary FITC-Fab at 0°C in HEK293 cells. Internalization was initiated by shifting the temperature to 37°C for 1–30 min in Ab-free medium, and the pH_v was determined by FRIA. CD4TI-Ub reached early endosomes ($pH \sim 6.2$) in 15 min and lysosomes ($pH \sim 4.8$) during the subsequent 15-min chase (Figure 4b). CD4TI accumulated slowly in recycling endosomes, consistent with its reduced internalization rate (Barriere *et al.*, 2006) and was detected there even after 90-min chase, presumably due to multiple endocytic and exocytic rounds (Figure 4b and not shown). Similar results were obtained when the primary and secondary Abs were internalized for 1 h and chased for 30 min at 37°C (Figure 4c), a protocol used in subsequent studies.

Poly-Ub and Multimeric-Ub Are Recognized as Lysosomal Sorting Signals at Early Endosomes

To investigate Ub configuration(s) that may serve as an efficient lysosomal-targeting signal, the postendocytic trafficking of chimeras exposing poly-, multimeric-, or mono-Ub was compared with the labeling protocol described in Figure 4c. Although internalized CD4TI-Ub was cleared from early endosomes and delivered to lysosomes during 30-min chase, CD4TI-UbRΔG harboring a single Ub moiety was remained in a compartment with characteristic pH_v of sorting endosomes (pH_v 6.56 $\pm$ 0.08; Mukherjee *et al.*, 1997) and was unable to reach the lysosome even after 90-min chase in both COS-7 and HEK293 cells (Figure 5a and Supplementary Table S1). CD4TI-UbR also accumulated, predominantly, in

early endosomes ($pH_v = 6.01 \pm 0.05$; Figure 5a and Supplementary Table S1). The limited lysosomal accumulation ($\sim 10\%$) of CD4TI-UbR indicated by the bimodal pH_v distribution could be explained by the recruitment of the ubiquitination machinery via the terminal Gly residue that may contribute to lysosomal sorting (Hochstrasser, 2006). The modest delay of CD4TI-UbΔG lysosomal delivery is consistent with this hypothesis (Figure 5a).

Mono-Ub was unable to function as an efficient lysosomal-sorting signal when it was fused to the truncated variant of the trimeric invariant chain, lacking endogenous internalization signals (Bonifacino and Traub, 2003; Figure 5b). Although IiT-UbRΔG accumulated at mildly acidic (pH_v 6.57 $\pm$ 0.03) compartment, lysosomal delivery of IiT-Ub was completed during the 30-min chase (Figure 5b). Thus the inability of mono-Ub to be recognized as a lysosomal-sorting signal is independent of the reporter protein and was also confirmed with CD4 as cargo (see Supplementary Figure S4).

Remarkably, multimerization of CD4TI-UbRΔG by two different means restored the chimera lysosomal delivery. Insertion of the cc tetramerization domain targeted CD4cc-UbRΔG and CD4cc-Ub similarly to CD4cc-UbR (data not shown) during the 30-min chase to highly acidic vesicles (pH_v 4.94 $\pm$ 0.09, 4.82 $\pm$ 0.14, and 4.88 $\pm$ 0.16, respectively; Figure 5c and Supplementary Table S1). In contrast, CD4cc was confined to recycling endosomes (pH_v 6.46 $\pm$ 0.09, $n = 3$), ruling out the role of the cc domain as a lysosomal-sorting motif (Figure 5c). As a second technique, cross-linking of CD4TI-UbRΔG with primary Ab, biotinylated secondary Ab, and streptavidin was used to

Figure 5. Lysosomal sorting of CD4-Ub chimera requires poly-Ub or multimeric-Ub. (a) Postendocytotic sorting of the indicated CD4 chimera in COS-7 cells was monitored by FRIA as described in Figure 4c. Data are expressed as frequency of pH_v and means of $pH_v \pm SEM$ from a single experiment (see also Supplementary Table S1). (b) Lysosomal sorting of invariant chain-Ub chimeras. The postendocytotic trafficking of the truncated invariant chain (IIT) lacking its internalization motif, IIT-Ub and IIT-UbRΔG was determined by the pH_v measurement using primary anti-Ii- and FITC-conjugated secondary Fab fragment with FRIA as described in *Materials and Methods*. (c) The pH_v of internalized CD4cc-, CD4cc-Ub-, and CD4cc-UbRΔG-containing vesicles was measured as in Figure 4c. (d). Multimerization of CD4TI and CD4TI-UbRΔG was achieved by incubating the cells sequentially with anti-CD4 Ab (OKT4), biotinylated secondary Ab and streptavidin-FITC on ice. Internalization was initiated at 37°C in Ab-free medium for 30 min, and pH_v was measured by FRIA. (e) Postendocytotic sorting of CD4cc-UbRI44A and CD4cc-UbR was established by pH_v measurements as in panel a.

induce noncovalent clustering of the chimera. This method also rerouted the chimera, but not CD4TI, from recycling endosomes into lysosomes (Figure 5d), implying that multimeric-Ub can be recognized as an efficient lysosomal-sorting signal.

Ub44Ile Is Critical for Lysosomal Sorting of CD4cc-UbRΔG

The hydrophobic patch around the Ub44Ile is required as the binding surface for Ub-binding domains, including the UIM of Hrs, STAM, epsin, and eps15 (Hicke, 2001; Fisher *et al.*, 2003; Hicke and Dunn, 2003). To demonstrate the relevance of UIM-Ub interaction in the lysosomal targeting of the CD4cc-UbRΔG, Ub44Ile was mutated to Ala. According to pH_v measurements, the CD4cc-UbRI44A was routed to recycling endosomes, in contrast to its wt counterpart, (pH_v 6.38 ± 0.10 , $n = 3$, Figure 5e). Concomitantly, the Ile44Ala mutation also restored the cell surface stability and recycling of the CD4cc-UbRI44A (Figure 2, a and c). These results confirm the inference that recognition of multiple Ub moieties is required for efficient lysosomal targeting and preventing constitutive recycling that leads to the metabolic destabilization of reporter molecules at the cell surface.

Inactivation of the E1 Enzyme Impedes the Lysosomal Delivery of the CD4TI-Ub

To demonstrate that ubiquitin conjugation is indeed required for the lysosomal sorting of the CD4TI-Ub, we took advantage of the ts20 cell line harboring a temperature-

sensitive E1 Ub-activating enzyme. Complete down-regulation of the thermosensitive E1 enzyme could be achieved at 40°C in 3 h, verified by immunoblotting (Figure 6a). Ub conjugation was indispensable for the CD4TI-Ub lysosomal targeting, because heat inactivation of the E1 enzyme dramatically impeded lysosomal accumulation of CD4TI-Ub in ts20 cells (Figure 6b, top panel). In contrast, 40°C exposure had no effect on the lysosomal delivery in E36 cells containing the wild-type E1 enzyme (Figure 6b, top panels). Notably, efficient lysosomal targeting of the tetrameric CD4cc-UbRΔG was preserved at the nonpermissive temperature in ts20 cells, indicating that ubiquitination of the sorting machinery is not essential for ubiquitinated cargo sorting (Figure 6b, bottom panels).

K63-linked Ub Chain as a Lysosomal Targeting Signal

In an attempt to elucidate the poly-Ub chain configuration that is recognized as a lysosomal-sorting signal, a single Lys residue was reinserted at position 6, 29, 48, or 63 into the UbRΔG. Lysosomal targeting of the CD4TI-UbRΔG63K was partially restored during 30-min chase, because ~58% of cargo reached the lysosomes (Figure 7a). Lysosomal delivery was less efficient for the 48K, reflected by ~21% lysosomal accumulation of intracellular cargo and it was not detectable for the 6K or 29K substitutions (Figure 7a). Saturation of the endosomal sorting machinery cannot explain the recycling of UbRΔG6K, UbRΔG29K, and UbRΔG48K, because the cell surface density of the respective chimeras was increased by about fourfold, whereas their internalization was attenuated

Figure 6. Inactivation of the E1 enzyme impairs Ub chain formation and lysosomal sorting of the CD4TI-Ub, but not the CD4cc-Ub Δ G. (a) Thermoinactivation of the E1 enzyme was accomplished at 40°C in ts20, but not in E36 cells. Equal amounts of cell lysates were probed with anti-E1 Ab using ECL. (b) Ts20 and E36 cells, expressing the indicated construct, were incubated at 40°C for 3 h to down-regulate the E1 Ub-activating enzyme. The pH_v of internalized CD4TI-Ub and CD4cc-Ub Δ G was determined by FRAP.

by ~75% relative to CD4TI-Ub (Supplementary Figure S1b). Thus K63 and K48 appear to serve as preferential acceptor sites for the first Ub conjugation.

To restrict the Ub chain configuration on CD4TI-Ub, the chimera was coexpressed with HA-tagged mutant Ubs, containing a single Lys (UbR63K or UbR48K) or no Lys residue (UbR). It was anticipated that overexpression of UbR terminated Ub chain extension, whereas UbR63K and UbR48K only restricted the chain configuration to K63- and K48-linked poly-Ub, respectively. The comparable expression and conjugation of Ub variants to cellular proteins was visualized by immunoblotting the HEK293 cell lysate with anti-HA Ab (Figure 7b). Similar results were obtained in COS-7 cells (data not shown). pH_v measurements verified that overexpression of UbR, but not wt Ub, prevented the lysosomal delivery of CD4TI-Ub during the 30–90-min chase (pH_v ~6.5, Figure 7c), conceivably by terminating chain extension. Neither wt nor UbR had discernable effects on Tf receptor recycling and Lamp1 or CD4cc-Ub Δ G lysosomal sorting, ruling out nonspecific effects of Ub overexpression on lysosomal sorting (Supplementary Figure S2a).

Although complete lysosomal accumulation of CD4TI-Ub was achieved in the presence of UbR63K after 30-min chase, at least 90 min was required in case of UbR48K expression (Figure 7c). Considering the comparable expression level of Ub variants (Figure 7b and Supplementary Figure S2) and their conjugation to CD4TI-Ub (Supplementary Figure S2), the simplest explanation is that the endosomal sorting machinery preferentially, but not exclusively, recognizes K63-linked Ub chain.

Preferential Binding of Hrs to K63-linked Polyubiquitin Chain

Hrs, a member of the ESCRT0, has been shown to play a pivotal role in ubiquitinated cargo recognition and lysosomal sorting at early endosomes (Hicke and Dunn, 2003; Raiborg *et al.*, 2003). Inhibition of Hrs function delays lysosomal degradation of numerous ubiquitinated membrane proteins (Bache *et al.*, 2003). Interaction of ubiquitinated CD4TI-Ub with Hrs was demonstrated by coimmunoprecipitation. According to immunoblot analysis, the immunoprecipitates of CD4TI-Ub, but not CD4TI, contained Hrs (Figure 8a and data not shown). Furthermore, Hrs down-regulation by specific small interfering RNA (siRNA) inhibited the lysosomal delivery of CD4TI-Ub, confirming Hrs involvement in the postendocytic sorting of CD4TI-Ub (data not shown).

To investigate the Ub-binding specificity of Hrs, we used GST fusion proteins containing 1–4 tandem Ub moieties, mimicking the linear configuration of the K63-linked Ub chain (Hofmann and Pickart, 2001). Although Hrs was recovered with GST-2Ub, GST-3Ub, and GST-4Ub pulldown, minimal or no endogenous Hrs was associated with GST-Ub (Figure 8b). Eliminating one of the UIM recognition sites by Ile44Ala substitution in GST-Ub-Ub1A was sufficient to inhibit Hrs binding (Figure 8b, lanes 3 and 7). Increasing the distance between two Ub moieties by a flexible linker (GST-Ub-L-Ub) was permissive for Hrs binding and rendered the fusion protein accessible to the E1-activating enzyme (Figure 8b, lanes 3 and 6). Notably, the E1 Ub-activating enzyme was also bound to GST-Ub, implying that misfolding of the GST-Ub cannot account for the lack of its Hrs binding (Figure 8b, middle panel). In light of the dimerization propensity of the GST, these results support the notion that multivalent interactions are necessary for Hrs binding to ubiquitinated polypeptides. Homo- and hetero-oligomerization of Hrs with a subset of Ub-binding adaptors (e.g., STAM, eps15, and TSG101) may account for this phenomenon at early endosomes (Bache *et al.*, 2003).

To assess the recombinant Hrs binding specificity, the association of mono-Ub, K63-, and K48-linked Ub chains with Hrs fused to maltose-binding protein (MBP) was determined *in vitro*. Comparable amounts of recombinant MBP-Hrs was bound to amylose beads and incubated with mono-Ub, K63-, and K48-linked Ub chains. Bound Ub was visualized by immunoblotting with anti-Ub Ab. Although neither the full-length nor a C-terminally truncated Hrs (Hrs298X) containing the UIM was able to bind detectable mono-Ub, a substantial amount of K63-linked poly-Ub chain was pulled down (Figure 8c). The recovery of K48-linked Ub chain was significantly attenuated (Figure 8c). Furthermore, disrupting Ub recognition by the S270A mutation in the UIM of Hrs (Miller *et al.*, 2004), significantly diminished Ub binding (Figure 8c). These *in vitro* observations are consistent with preferential recognition of the K63-linked Ub chain by the Ub-dependent endosomal sorting machinery *in vivo*.

Lysosomal Sorting Signal of MARCH-IV Ubiquitinated CD4

Herpes viruses have been shown to encode viral homologues of mammalian proteins. Several candidates for mammalian homologues of modulator of immune recognition (MIR) family members share catalytic domains with viral E3 Ub ligases (Coscoy and Ganem, 2000; Coscoy *et al.*, 2001). Based on their structural characters, MIRs have been designated as MARCH family members (Bartee *et al.*, 2004). The E3 ligase activity of MARCH-IV ubiquitinates and down-

Figure 7. The effect of Ub chain configuration on the internalization and postendocytic sorting of CD4TI-Ub chimeras. (a) Postendocytic sorting of CD4TI-UbRΔG6K, -UbRΔG29K, -UbRΔG48K, and -UbRΔG63K was monitored by FRIA in transiently transfected COS-7 cells as in Figure 4c. The vesicles displaying the larger cohort of pH_v have gray shade. (b) Immunoblot analysis of COS-7 cells transiently transfected with HA-tagged Ub variants. Equal amounts of cell lysates were separated by SDS-PAGE and probed with anti-HA Ab. (c) Transient coexpression of UbR, but not wt Ub, prevents lysosomal accumulation of CD4TI-Ub. The postendocytic fate of chimeras was monitored by pH_v as in Figure 4c in COS-7 cells transiently coexpressing CD4TIUb and Ubs variants at a plasmid ratio of 1:3–4. (d) The postendocytic sorting of CD4TI-Ub in the presence of overexpressed UbR63K or UbR48K was monitored by pH_v. When indicated, the chase was extended to 90 min. Similar results were obtained in HEK293 cells stably expressing CD4TI-Ub and transfected with plasmids encoding the Ub variant and the dsRed fluorescent protein at a ratio of 10:1. FRIA was performed on cell with dsRed expression. (e) Internalization rates of CD4TI-Ub in the presence of overexpressed Ub variant. Endocytic rates were measured by the anti-CD4 Ab uptake assay in transiently cotransfected COS-7 cells as described in *Materials and Methods*. Means ± SEM; n = 3; *p > 0.05 relative to mock transfected CD4TI-Ub.

regulates CD4 from the cell surface (Mansouri *et al.*, 2003). To examine the relevance of Ub chain topology in MARCH-IV-induced CD4 down-regulation, three of four Lys residues was eliminated in the cytoplasmic tail of CD4 to prevent multiple Ub-chain formation (see *Materials and Methods*). In addition, we mutated the di-Leu motif to minimize Ub-independent sorting of CD4.

According to pH_v measurements, CD4-1K was confined to recycling endosomes in the absence of MARCH-IV, whereas overexpression of MARCH-IV provoked the lysosomal delivery of CD4-1K during a 45-min chase (Figure 9a). Notably, the Lys-less CD4 (CD4-4R) variant that was inaccessible to ubiquitination remained at early endosomes even after 90-min chase (Figure 9A). Likewise, no lysosomal delivery of the CD4-1K was observed upon coexpression of MARCH-II that failed to down-regulate the CD4 (Bartee *et al.*, 2004; data not shown).

Preventing poly-Ub chain formation by UbR overexpression inhibited the MARCH-IV-induced lysosomal delivery of the

CD4-1K (Figure 9b). On the other hand, overexpression of UbR63K supported, whereas UbR48K delayed lysosomal arrival of the CD4-1K compared with that in the presence or absence of wt Ub coexpression (Figure 9, a and b). Intriguingly, similar sensitivity was documented for the lysosomal-targeting efficiency of the full-length CD4, probed also by coexpression of wt Ub, UbR, UbR63K, and UbR48K (Supplementary Figure S4). These observations suggest that only one of the Lys residues is susceptible to MARCH-IV-dependent ubiquitination and are in accord with the polyubiquitination requirement of CD4 for efficient lysosomal sorting.

DISCUSSION

Taking advantage of model proteins with distinct Ub configuration and the MARCH-IV-dependent ubiquitination of CD4, we determined the Ub configuration that is necessary and sufficient for efficient lysosomal sorting of internalized transmembrane cargo. Using immunocytochemical, phar-

Figure 8. Ub binding specificity of endogenous and recombinant Hrs. (a) Association of Hrs with CD4TI-Ub in HEK293 cells. Immunoprecipitated CD4TI-Ub with the OKT4 anti-CD4 Ab was probed with the indicated Ab. One hundred micrograms of lysates (lys) was loaded. (b) Equal amounts of GST fusion proteins containing one, two, three, or four tandem Ubs were incubated with HeLa cell lysate as described in *Materials and Methods*. Bound Hrs (top panel) and E1 Ub-activating enzyme (middle panel) was visualized by immunoblotting. The mutant variants of GST-2Ub are described in *Materials and Methods*. Ten percent of lysate (lys) was loaded. GST-Ub fusions are visualized by Ponceau staining (bottom panel). (c) Preferential binding of K63-linked Ub-chain binding to recombinant Hrs. Similar amounts of MBP, wt, or mutant MBP-Hrs fusion proteins were bound to amylose beads and incubated with K48- (left panel), K63-linked poly-Ub chain (middle panel), or mono-Ub (right panel). Bound Ub was visualized by immunoblotting, using the P4D1 anti-Ub Ab. Ubs were also loaded for direct comparison as specified in *Materials and Methods*.

macological, and functional approaches, as well as FRiA to follow cargo trafficking by vesicular pH measurements in live cells, we provide compelling evidence showing that both poly-Ub and multimeric-Ub, but not monomeric Ub, constitute an efficient lysosomal-targeting signal for endocytosed transmembrane proteins. The preferential binding of poly-Ub chain to endogenous and recombinant Hrs in vitro and in vivo is in line with the functional data.

The Relevance of Polyubiquitination as a Lysosomal Targeting Signal

Although monoubiquitination was originally discovered as an endocytic signal of plasma membrane receptors and transporters (Ste2p, or galactose Gal2p and maltose transporters) in yeast (Lucero and Lagunas, 1997; Terrell *et al.*, 1998; Horak and Wolf, 2001), recent analysis revealed that multiple Ub attachment can trigger more efficient cargo internalization than mono-Ub (e.g., Ste2p, the a-factor receptor Ste3p, the a-factor transporter Ste6p and the zinc transporter; Kolling and Hollenberg, 1994; Galan and Hagenauer-Tsapas, 1997; Terrell *et al.*, 1998; Gitan and Eide, 2000; Roth and Davis, 2000). In animal cells poly- or multimeric-Ub, but not unextendable Ub modification was required to signal rapid endocytosis of model proteins (Barriere *et al.*, 2006; Hawryluk *et al.*, 2006). In accord, poly- or multiple-mono ubiquitination of numerous plasma membrane proteins was documented to be involved in the regulation of their cell surface density (for a review, see Traub and Lukacs, 2007). Ligand-induced down-regulation of TrkA receptor, as well as the MIR K3-mediated MHC I ubiquitination requires K63-linked poly-Ub chain formation (Duncan *et al.*, 2006; Geetha *et al.*, 2005). On the other hand, the activated dimeric tyrosine kinase receptor (e.g., EGFR)

undergoes both multiple mono- and poly-ubiquitination (Haglund *et al.*, 2003; Geetha *et al.*, 2005). Polyubiquitination of MHC II complex is also critical in limiting antigenic peptide presentation (Ohmura-Hoshino *et al.*, 2006; Shin *et al.*, 2006; van Niel *et al.*, 2006). Virally encoded E3-Ub ligases of the MIR family polyubiquitinates numerous immune recognition molecules, including B7.2, ICAM-I, CD1d, CD4, and interferon γ receptor 1 (Lehner *et al.*, 2005; Li *et al.*, 2007). Polyubiquitination of channels, transporters, and other receptors (AQP2, ENaC, β_2 adrenergic receptor-arrestin complex, TRPV4, CFTR, and CIC-5) is also indispensable for internalization (Staub *et al.*, 1997; Shenoy *et al.*, 2001; Hryciw *et al.*, 2004; Sharma *et al.*, 2004; Kamsteeg *et al.*, 2006; Wegierski *et al.*, 2006; Wiemuth *et al.*, 2007). These observations, collectively, highlight the prevalence and functional significance of poly- and multiple-mono ubiquitination as autonomous endocytic signal and reinforce their relevance as possible lysosomal-targeting signal.

Monitoring Polyubiquitination or Multiple-mono Ubiquitination Requirement for Lysosomal Cargo Sorting by Vesicular pH Determination

Considering that cargo ubiquitination signals both internalization and lysosomal targeting from early endosomes and accelerated internalization per se could lead to enhanced endolysosomal cargo flux (Bonifacio and Traub, 2003), it was imperative to design an assay that monitors the dynamics of endocytosed cargo destination. This was achieved by implementing the FRiA assay to determine the postendocytic localization of cargo molecules based on the characteristic acidification pattern of endolysosomal compartments (Mukherjee *et al.*, 1997). Although sorting and recycling endosomes, as well as MVB and lysosomes have distinct

Figure 9. Polyubiquitination is required for CD4-1K lysosomal targeting. (a) Postendocytic sorting of CD4-1K and CD4-4R in the presence or absence of MARCH-IV and wt Ub was monitored by FRIL in transiently transfected COS-7 cells. Abs were internalized for 1.5 h and chased for the indicated time. (b) Postendocytic sorting of CD4-1K was monitored in the presence of MARCH-IV and UbR, UbR63K, or UbR48K as described in panel a. The chase period was 45 or 90 min.

luminal pH, the TGN pH ~6.2–6.6 overlaps with that of recycling endosomes (Sun-Wada *et al.*, 2004). Therefore, the recycling propensity of cargoes was also monitored by co-immunolocalization with the Tf receptor (TfR; Figure 3) and quantification of recycling efficiency by the biotin-avidin sandwich technique (Figure 2c).

Several lines of evidence support our conclusion that internalized model proteins and the CD4 require poly- or multimeric Ub modification for efficient MVB/lysosome targeting from endosomes. 1) Preventing Ub-chain conjugation by fusing unextendable Ub (UbRΔG) to the truncated CD4 or Li, similar to overexpression of UbR with CD4TI-Ub or CD4, was sufficient to reroute these cargoes to recycling endosomes, according to morphological, biochemical, and functional assays. On the other hand, constitutive recycling

of the CD4TI-UbRΔG could be converted into lysosomal targeting by introduction a single Lys residue (K63) into UbRΔG. The recycling propensity of CD4 chimeras was inversely proportional to their lysosomal-targeting efficiency, substantiating the role of endosomes in defining the destination of ubiquitinated cargo at sorting endosomes. 2) Thermodenaturation of the E1 Ub-activating enzyme severely inhibited the lysosomal delivery of the polyubiquitinated CD4TI-Ub in ts20 cells. This inhibition was specific, because E1 inactivation failed to interfere with the recycling of TfR and the lysosomal delivery of Lamp1 or CD4cc-UbRΔG, cargo molecules with ubiquitination-independent sorting signals. 3) Multimerization of monoubiquitinated CD4TI-UbRΔG, accomplished by genetic and biochemical means, restored the lysosomal targeting of chimera. This implies that the Ub-dependent endosomal sorting machinery has the plasticity to recognize both Ub chain and multimeric-Ub. The latter phenomenon has particular significance in the desensitization of activated dimeric tyrosine kinase receptors that undergo multiple mono- and polyubiquitination (Haglund *et al.*, 2003; Geetha *et al.*, 2005) and oligomeric polypeptides with monoubiquitinated subunits such as the ROMK1 K⁺ channel (Lin *et al.*, 2005). Notably, constitutive trimerization failed to redirect the Li-UbRΔG to the lysosome, whereas tetrameric mono-Ub was rapidly delivered into MVB/lysosomes in the context of CD4cc-UbRΔG. This suggests that exposure of at least four Ub moieties is required for lysosomal sorting, although we cannot rule out the possibility that trimerization masked the UIM binding surface in the Li-UbRΔG. 4) Finally, we showed that disrupting the Ub-UIM-interacting surface by the UbL44A mutation prevented the CD4Tcc-UbRΔG MVB/lysosomal sorting and restored recycling, corroborating the notion that Ub-binding proteins are critical in the lysosomal sorting of chimeras.

Recognition of Poly- and Multiubiquitinated Cargo in Early Endosomes

The observation that the Ub-dependent endosomal sorting machinery preferentially recognizes poly- and multiubiquitinated over monoubiquitinated cargo is not completely surprising in light of biochemical and functional characteristics of Ub recognition as an endocytic signal (Williams and Urbe, 2007). Although decoding Ub as a sorting motif relies on different sets of adaptors at various cellular locations, the primary recognition of the Ub Ile44 hydrophobic patch is mediated by a common α-helical Ub-binding domain, the UIM of adaptors both at the cell surface and at sorting endosome (Hicke *et al.*, 2005; Hurley and Emr, 2006). Eps15/15R and epsin, plasma membrane clathrin adaptors, contain two and three UIMs, respectively (Bonifacino and Traub, 2003; Hicke and Dunn, 2003). The endosomal adaptor STAM has a single UIM, whereas Hrs contains the double-sided variant of UIM, the DUIM, which is capable of binding two Ubs simultaneously with affinities comparable to that of the UIM (Urbe *et al.*, 2003; Hirano *et al.*, 2006). In vitro-binding studies revealed that the UIMs bind to mono-Ub with a surprisingly low affinity in the 0.1–2 mM range (Shekhtman and Cowburn, 2002; Fisher *et al.*, 2003). This may explain the inability of mono-Ub to serve as internalization (Barriere *et al.*, 2006) and lysosomal-sorting signal for transmembrane cargo in mammalian cells (Duncan *et al.*, 2006). Synergistic mechanisms are likely involved to enhance the low-affinity UIM-Ub interaction in vivo. These may include multimerization of adaptor molecules, multiplication of Ub-binding domains in adaptors, concentration of adaptor/cargo at specific sorting platforms, and modulation of UIMs affinity

(Cupers *et al.*, 1997; Chen *et al.*, 1998; Raiborg *et al.*, 2002; Sachse *et al.*, 2002; Bache *et al.*, 2003; Sugiyama *et al.*, 2005; Hurley and Emr, 2006).

In respect of Ub recognition as a lysosomal-sorting signal, stable complex formation between Hrs and STAM (also termed ESCRT0) can facilitate the binding of multiple Ub moieties. The yeast orthologues of Hrs and STAM–Vps27 and Hse1–contain two tandem and a single UIM, respectively (Hurley and Emr, 2006; Williams and Urbe, 2007). The Vps27/Hse1 complex associates with multiple Ubs, situated at different distances from the membrane according to Monte Carlo simulation and can undergo large conformational changes to accommodate multiple Ub moieties (Prag *et al.*, 2007). Considering that the VHS domain of STAM can also recognize Ub (Mizuno *et al.*, 2003), heterodimerization of Hrs/STAM would expose four Ub-binding sites. Furthermore, the oligomerization tendency, indicated by the hexamerization of recombinant Hrs (Pullan *et al.*, 2006) and isolation of endogenous Hrs in 500–600-kDa complexes from HeLa cell lysate (unpublished observation), may further enhance the number of functional Ub-binding sites in ESCRT0. Finally, concentration of both ubiquitinated cargo and the Hrs/STAM complex at the endosomal double-layered coat by clathrin recruitment and PtdIn3P binding of the Hrs FYVE domain could increase the association of the ubiquitinated cargo and the ESCRT0 (Sachse *et al.*, 2002). Remarkably, homo- or hetero-oligomerization of eps15/15R/epsin adaptors may also serve to enhance their avidity to poly-Ub chain (Cupers *et al.*, 1997; Chen *et al.*, 1998; Polo *et al.*, 2002; Miller *et al.*, 2004; Sugiyama *et al.*, 2005; Hawryluk *et al.*, 2006).

We showed that the K48-linked poly-Ub chain is poorly recognized by recombinant Hrs *in vitro*, despite the 2–3-fold slower lysosomal-targeting kinetics of the CD4TI-Ub in the presence of overexpressed UbR48K (Figures 7d and 9b, and Supplementary Figure S4). These observations are at variance with the negligible effect of UbR48K and UbR63K overexpression in the internalization of the CD4TI-Ub (Figure 7e), but in line with the modest Ub-chain selectivity of the epsin UIMs (Wang and Struhl, 2005; Hawryluk *et al.*, 2006). The relatively limited Ub-chain specificity of the Ub-dependent endosomal-sorting machinery compared with recombinant Hrs could be explained by a combination of factors. The substrate specificity of Hrs could be altered by association with STAM, clathrin heavy chain, and PtdIns3P (Williams and Urbe, 2007). Furthermore, posttranslational modification, such as monoubiquitination and phosphorylation of Hrs/STAM, may play a role in the ESCRT0 binding specificity *in vivo* (Row *et al.*, 2005; Hoeller *et al.*, 2006). Finally, decreased availability of STAM due to K48 poly-Ub chain conjugation and proteolysis via proteasomes may influence lysosomal-sorting kinetics of ubiquitinated cargo (Williams and Urbe, 2007).

Modulation of Lysosomal Sorting Fidelity by the Extent of Poly- and Multiubiquitination of Cargo Molecules

We envision two advantages of poly- and multimeric-Ub as a lysosomal-sorting signal over mono-Ub. First, the redundant nature of ubiquitin modification may permit flexibility for substrates and E2/E3 enzyme pairing, as well as for the Ub chain configuration. Although some plasma membrane proteins undergo K63-linked Ub chain attachments, both K63- and K48-linked Ub chains were detected in activated EGFR (Huang *et al.*, 2006). Preferential K48-linked over K63-linked Ub chain conjugation would delay the desensitization of activated receptors, based on the significantly slower lysosomal transfer kinetics of cargoes with K48-lined Ub

chain. Second, attachment of Ub chain or multimeric-Ub may allow fine-tuning of the sorting efficiency by regulating signal recognition via the opposing activity of Ub ligase(s) and deubiquitinating enzyme(s) (DUB) at endosomes (Amerik and Hochstrasser, 2004) as opposed to the bimodal regulation with a single Ub-sorting motif. This modulation would be conceptually similar to the role that ligases and deubiquitinating enzymes play in influencing the destruction of polyubiquitinated polypeptides by the proteasome (Crosas *et al.*, 2006).

Association of DUB and E3 Ub ligase with constituents of the ESCRT0 have been documented in both yeast and mammalian cells (Clague and Urbe, 2006). In yeast, the Vps27/Hse1 complex binds to the DUB Ubp7, as well as the Rsp5 ligase to control sorting efficiency into vacuoles (Ren *et al.*, 2007). In mammalian cells, the SH3 domain of STAM recruits UBPY (ubiquitin isopeptidase Y; Kato *et al.*, 2000) and AMSH (associated molecule with SH3 domain of STAM; McCullough *et al.*, 2006). The isopeptidase activity of UBPY cleaves both the K63- and K48-linked Ub chains, whereas AMSH shows preferential activity toward the K63-linked Ub chain. Thus AMSH serves as an off switch, whereas UBPY, depending on configuration of the primary Ub chain and on the E3 ligase activity in the sorting complex, may accelerate or delay lysosomal cargo delivery.

Although our FRiA experiments allowed us to follow the endosomal sorting of internalized ubiquitinated cargo molecules and highlighted the necessary and sufficient Ub configuration for lysosomal targeting, the cellular location and the molecular determinants of cargo-specific ubiquitination remains to be established. Nevertheless, the FRiA technique complements existing methodologies to follow the destination of endocytosed plasma membrane proteins at relatively high temporal and spatial resolution and will help to elucidate the molecular basis for the recognition of sorting signals at endosomes.

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