Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis

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Leptin receptors are constitutively endocytosed in a ligand-independent manner. To study their endocytosis, leptin receptors OB-Ra and OB-Rb were expressed in HeLa cells. Both receptor isoforms were ubiquitylated, internalized by clathrin-mediated endocytosis and transported to Hrs-positive endosomes after their internalization. Proteasome inhibitors inhibited OB-Ra but not OB-Rb internalization from the cell surface. OB-Ra ubiquitylation occurred on lysine residues K877 and K889 in the cytoplasmic tail, the mutation of which abolished OB-Ra internalization. Fusion of an ubiquitin molecule at the C-terminus of an OB-Ra construct defective both in ubiquitylation and endocytosis restored clathrin-dependent endocytosis of the receptor. The internalization of this constitutively mono-ubiquitylated construct was no longer sensitive to proteasome inhibitors, which inhibited OB-Ra endocytosis by blocking its ubiquitylation. Fusion of an ubiquitin molecule to a transferrin receptor deleted from its own endocytosis motif restored clathrin-mediated endocytosis. We propose that mono-ubiquitin conjugates act as internalization motifs for clathrin-dependent endocytosis of leptin receptor OB-Ra.

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Introduction

Endocytosis of many cell surface receptors and membrane proteins begins with their selective recruitment in clathrin-coated pits. Interaction between endocytosis motifs of membrane proteins that are to be internalized and adaptors of the clathrin-coated pits are thought to play an essential role in the initiation of this process (Schmid, 1997; Hirst and Robinson, 1998; Kirchhausen, 2000). Several types of endocytosis motifs have been identified in membrane proteins. The best characterized of these are the dileucine and tyrosine-based motifs (Bonifacino and Traub, 2003).

In the yeast *Saccharomyces cerevisiae*, ubiquitin molecules conjugated to lysine residues of membrane proteins function

as endocytosis motifs (Hicke and Dunn, 2003). In contrast to cytosolic and nuclear proteins that are targeted to degradation via the proteasome by the addition of polyubiquitin chains, membrane proteins usually are modified by mono- or di-ubiquitin conjugates. Besides its role in endocytosis, the ubiquitylation of yeast membrane proteins is essential for their sorting to internal vesicles of multivesicular bodies, en route to the vacuole. In mammalian cells, ubiquitin conjugation plays a similar role in the endosomal sorting of membrane proteins. Multiple proteins that function in ubiquitin-dependent endosomal sorting have now been identified (Katzmann *et al*, 2002; Marmor and Yarden, 2004). However, evidence is still missing that ubiquitin functions as internalization motif in mammalian cells. In-frame fusion of ubiquitin to membrane proteins results in constitutive internalization (Nakatsu *et al*, 2000; Haglund *et al*, 2003; Mosesson *et al*, 2003), indicating that mono-ubiquitylation is sufficient to induce internalization. Nevertheless, the mutation of all ubiquitin-acceptor lysine residues of CXCR4 or of the growth hormone receptor (GHR), two proteins that are normally ubiquitylated at the plasma membrane, did not inhibit their internalization (van Kerkhof *et al*, 2000; Marchese and Benovic, 2001). Also consistent with the notion that ubiquitylation may not be necessary for clathrin-mediated endocytosis, the rate of ligand-induced internalization of EGF receptor is very similar in Cbl-deficient and control cells, despite the lack of EGF receptor ubiquitylation in Cbl-deficient cells (Duan *et al*, 2003). Similar findings were reported with dominant-negative mutants of Cbl (Levkowitz *et al*, 1998; Thien *et al*, 2001). In all these reports, the inhibition of ubiquitylation impaired endosomal sorting and lysosomal degradation, but not the internalization step from the cell surface.

In this paper, we show ubiquitin-dependent endocytosis of OB-Ra, a leptin receptor. Leptin is a polypeptide hormone secreted by adipocytes that is a key regulator of energy balance, and neuroendocrine functions (Friedman and Halaas, 1998; Ahima and Flier, 2000). Several isoforms of leptin receptor (OB-R), including the two main isoforms OB-Ra and OB-Rb originate from a unique gene by alternative splicing, and are expressed in the central nervous system and in many peripheral tissues (Friedman and Halaas, 1998; Ahima and Flier, 2000). OB-Rs are type I glycoproteins structurally related to class I cytokine receptors (Tartaglia, 1997). All OB-R isoforms are identical in their N-terminal extracellular domain, transmembrane domain and in their cytoplasmic domain up to lysine 889, but differ in the C-terminus of their cytoplasmic tails. OB-Rb, also referred to as the long isoform, is responsible for the transduction of leptin signaling, whereas the function of OB-Ra is unknown. OB-Ra and OB-Rb are short-lived proteins that are constitutively endocytosed in a ligand-independent manner (Belouzard *et al*, 2004). After being internalized, they do not appear to recycle, but are transported through the endocytic pathway to lysosomes, where they are degraded

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with their ligand (Barr *et al*, 1999; Uotani *et al*, 1999). The cytoplasmic tail is necessary for OB-R internalization (Uotani *et al*, 1999; Belouzard *et al*, 2004); however, OB-Ra cytoplasmic tail contains no tyrosine or dileucine-based endocytosis motif, indicating that OB-Ra cytoplasmic tail carries a new type of endocytosis motif.

Results

Leptin receptors are internalized by clathrin-mediated endocytosis

Leptin receptors are constitutively endocytosed in a ligand-independent manner (Belouzard *et al*, 2004). To assess the role of clathrin in this process, we knocked down clathrin heavy chain (CHC) using siRNA. Following two successive transfections of siRNA, CHC levels were downregulated to about 20% of controls (Figure 1A). HA-tagged OB-Rs were expressed in siRNA-treated HeLa cells, and their internalization was evaluated by antibody uptake. HeLa cells expressing OB-Rs were incubated for 10 min at 37°C with anti-HA MAb to label internalized receptors. The cells were fixed and processed for immunofluorescent detection of anti-HA antibody. As shown in Figure 1B, OB-R internalization was dramatically impaired in most of the cells that were treated with CHC siRNA, whereas control siRNA had no detectable effect on OB-R endocytosis. Both receptors were detected in a perinuclear endosomal compartment in cells treated with control siRNA, whereas they were mainly located at the cell surface in CHC siRNA cells (Figure 1B). In comparison, the uptake of Alexa555-conjugated transferrin was also monitored. As expected for a clathrin-dependent cargo, CHC siRNA had a strong inhibitory effect on transferrin internalization. Similar levels of inhibition were achieved for leptin and transferrin receptors (Figure 1C). These results suggest that an active clathrin-mediated pathway of endocytosis is required for OB-Ra and OB-Rb to be internalized.

Internalized leptin receptors are localized in Hrs-positive endosomes

After internalization from the cell surface, OB-Rs are eventually transported to, and degraded in lysosomes (Barr *et al*, 1999; Belouzard *et al*, 2004). However, the intracellular trafficking of internalized OB-R has not yet been investigated. As shown in Figure 1, OB-Rs were located in perinuclear endosomes after internalization. In order to further characterize OB-R endocytic pathway, the intracellular localization of OB-R after internalization from the cell surface was examined by confocal microscopy. OB-R-positive perinuclear endosomes did not colocalize with transferrin receptor (TfR) labeling (Figure 2). Moreover, OB-R-positive endosomal compartment was insensitive to brefeldin-A (BFA) treatment, in contrast to TfR-positive perinuclear endosomes, on which a 5-min treatment with BFA induced an extensive tubulation in HeLa cells (data not shown). These results indicate that OB-R was not localized in TfR-positive recycling endosomes after their internalization from the cell surface.

OB-R-positive endosomes were further characterized with various markers of endocytic compartments. Initial experiments in HeLa cells indicated that internalized OB-Ra and OB-Rb were not colocalized with caveolin-positive compartments (data not shown) and that they were partially colocalized with Early Endosome antigen-1 (EEA-1) (Figure 2), and

Figure 1 Clathrin-dependent endocytosis of leptin receptors. HeLa cells were treated with control or clathrin siRNA and transfected with plasmid encoding HA-tagged OB-Ra or OB-Rb. (A) Anti-clathrin (top) or anti-actin (bottom) immunoblot of cell extracts. (B) Internalization of OB-Ra and OB-Rb in clathrin-depleted cells. Cells were incubated for 10 min with an anti-HA MAb at 37°C. Internalized antibodies were detected with cy3-conjugated secondary antibody after fixation and permeabilization. The internalization of transferrin receptor was monitored in parallel with Alexa555-conjugated transferrin (Tf). (C) Quantification of the internalization in control (white bar) or clathrin-depleted (gray bars) cells. Data are expressed as percentage of cells that internalized receptors.

Rab5-GFP (data not shown). This indicated that a part of internalized OB-R was located in early endosomes. At a higher magnification, internalized OB-R and EEA-1 were often found closely associated side by side, rather than really superimposed (Figure 2). Very similar patterns were obtained when internalized receptors were labeled with anti-HA MAbs or with Alexa555-conjugated leptin (Supplementary Figure S1), indicating that the antibodies did not cause a different receptor trafficking than the natural ligand. This pattern of close association could represent OB-R-positive endocytic

Figure 2 Characterization of leptin receptor-positive endosomes. HeLa cells (top rows) expressing HA-tagged OB-Ra or OB-Rb, and COS cells expressing HA-tagged OB-Ra (bottom rows) were allowed to internalize anti-HA MAb for 20 min and processed for double-label immunofluorescent detection of internalized MABs together with endosomal markers, as indicated. EEA-1, early endosome antigen-1; TfR, transferrin receptor; Hrs, Hepatocyte growth factor-regulated tyrosine kinase substrate.

carriers tethered to EEA-1 positive compartments, and ready to fuse with them. Alternatively, EEA-1 and OB-Rs could be localized on different membrane domains of single endosomes, as it was reported for other internalized membrane proteins (Raiborg *et al.*, 2002).

To test this possibility, OB-Ra was transfected in COS cells, which have larger endosomes than HeLa cells. The localization of internalized OB-Ra was compared to that of two markers, EEA-1 and Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), which are recruited on different membrane domains of early endosomes (Raiborg *et al.*, 2001). Again, a partial colocalization between internalized OB-Ra and EEA-1 was observed (Figure 2). At higher magnification, the pattern of labeling was consistent with both proteins being localized in different membrane domains of endosomes. In contrast, a much better colocalization was ob-

served with Hrs both at lower and higher magnifications (Figure 2). Taken together, these results suggest that after internalization from the cell surface OB-Rs are transported to early endosomes, where they localized in Hrs-coated membrane domains.

Proteasome inhibitors inhibit OB-Ra internalization from the cell surface

Hrs is known to interact with ubiquitylated membrane proteins in endosomes (Raiborg *et al.*, 2002). Therefore, the colocalization of Hrs and OB-Ra prompted us to investigate a role for ubiquitylation in OB-R endocytic trafficking. In order to examine a possible role for the ubiquitin/proteasome system in the regulation of OB-R degradation, we incubated OB-R-expressing cells in the presence of lactacystin, a potent and specific proteasome inhibitor, and analyzed the

expression levels of OB-Ra and OB-Rb by immunoblotting. A 2-h treatment with lactacystin increased OB-Ra and OB-Rb levels in cell lysates (Figure 3A). OB-R endocytosis was then analyzed by antibody uptake. Surprisingly, OB-Ra accumu-

Figure 3 Inhibition of OB-Ra endocytosis by lactacystin. (A) Stabilization of OB-R by lactacystin. HeLa cells expressing HA-tagged OB-Ra or OB-Rb were incubated in the presence (+), or the absence (–) of lactacystin for 2 h, and their content in OB-Ra or OB-Rb was analyzed by immunoblot. The lower band is not specific. The migration of prestained molecular weight markers is indicated on the left side of the gel. (B) Impact of lactacystin treatment on OB-R internalization. HeLa cells expressing HA-tagged OB-Ra or OB-Rb were incubated in the presence, or the absence of lactacystin for 2 h. OB-R endocytosis was analyzed by a 20-min anti-HA antibody uptake. (C) Inhibition of OB-Ra internalization by lactacystin. HeLa cells expressing HA-tagged OB-Ra were biotinylated at 4°C and allowed to internalize for 15 min at 37°C, or kept on ice (4°C). Endocytosed OB-Ra was protected from glutathione reduction (+ GSH), isolated using streptavidin beads and quantified by immunoblot using anti-HA, together with 20% of the proteins initially biotinylated and not submitted to glutathione reduction (–GSH). (D) Impact of lactacystin on steady-state OB-Ra localization. HeLa cells transiently expressing HA-tagged OB-Ra were incubated for 2 h in the presence (LC) or the absence (control) of lactacystin, and processed for immunofluorescence detection of OB-Ra.

lated at the cell surface in the presence of lactacystin (Figure 3B), or in the presence of MG132, another specific proteasome inhibitor (data not shown). Such a block in OB-Ra endocytosis was not observed after shorter treatments of the cells with proteasome inhibitors. In contrast, OB-Rb was internalized normally (Figure 3B). The inhibition of OB-Ra endocytosis was confirmed with a biochemical assay based on cell surface biotinylation and glutathione cleavage resistance. The levels of OB-Ra protected from glutathione cleavage after 15 min at 37°C decreased dramatically in lactacystin-treated cells, as expected for a protein that is not internalized from the cell surface (Figure 3C). Accordingly, the main steady-state intracellular localization of OB-Ra was shifted from perinuclear endosomes to the plasma membrane in lactacystin-treated cells (Figure 3D). This shift in steady-state intracellular localization is indicative of a strong inhibition of endocytosis. A similar redistribution was not visible for OB-Rb or TfR in lactacystin-, or MG132-treated cells (data not shown). These data suggest that proteasome activity is required either directly or indirectly, for OB-Ra but not for OB-Rb to be internalized from the cell surface.

Lysine residues K877 and K889 are essential for OB-Ra endocytosis

A well-defined function of the proteasome is to degrade cytosolic and nuclear proteins tagged with polyubiquitin chains. In addition, ubiquitin conjugation is also involved in the control of different steps of intracellular transport (Hicke and Dunn, 2003). Therefore, we sought to determine if OB-Ra could be endocytosed in an ubiquitylation-dependent manner. It is well established that ubiquitin is coupled to proteins on lysine residues. To inhibit any possible ubiquitylation of OB-Ra, all of the four lysine residues of its cytoplasmic domain were mutated into arginines (Figure 4A). The corresponding lysine-less mutant (4R) was expressed in HeLa cells. In the antibody uptake assay, no endosomes were labeled (Figure 4B), indicating that this mutant was impaired for endocytosis. We next mutated either lysines 866 and 867 (2R2K mutant), or lysines 877 and 889 (2K2R mutant) (Figure 4A). The 2R2K mutant was able to internalize antibodies in a manner very similar to OB-Ra, indicating that lysines 866 and 867 are not required for OB-Ra endocytosis. In contrast, only the cell surface was labeled with 2K2R (Figure 4B), indicating that at least one of the lysines 877 and 889 is necessary for OB-Ra to be internalized. Therefore, we generated two new constructs, one with the K877R mutation (2KRK mutant) and another one with the K889R mutation (3KR mutant) (Figure 4A). When expressed in HeLa cells, both of these constructs were found able to internalize antibodies (Figure 4B). This result indicated that either lysine 877 or lysine 889 is sufficient for OB-Ra endocytosis, and suggested that these two lysine residues fulfill similar or redundant functions.

To quantify the relative rate of endocytosis of each mutant, labeled endosomes were automatically counted in 120 randomly chosen cells from four independent transfections. The mean endosome number ranged from 8.98 for the 4R mutant to 82.15 for OB-Ra (Figure 4C). For comparison, the OB-RΔ1 mutant, which is deleted from its entire cytoplasmic domain and is not endocytosed (Belouzard *et al.*, 2004), gave a mean of 6.87 endosomes per cell (data not shown). The statistical analysis revealed that the endosome number per cell was not

significantly different between OB-Ra and 2R2K, and between 2K2R and 4R. 2KRK and 3KR were found significantly different from each other, and from all of the other constructs including OB-Ra. These data indicated that the mutations K877R (2KRK mutant) and K889R (3KR mutant) reduced the endocytosis rate of OB-Ra by about 14 and 32 %, respectively,

whereas the double mutation (2K2R mutant) almost completely blocked endocytosis.

The role of lysine residues K877 and K889 in OB-Ra endocytosis was confirmed with the glutathione cleavage resistance assay. The levels of receptor protected from glutathione cleavage after 15 min at 37°C were similar for OB-Ra and 2R2K, and lower in 4R-, and 2K2R-expressing cells (Figure 4D).

A

	866	867	877	889
OB-Ra	HQRMK	KLFWDDVP	NPKNCSWAQGLNFQ	KRTDTL
4R		RR	R	R
2R2K		RR	K	K
2K2R		KK	R	R
2KRK		KK	R	K
3KR		KK	K	R

Lysines K877 and K889 are ubiquitin-acceptor residues

To determine if leptin receptors actually are ubiquitinated, OB-Ra and OB-Rb were immunoprecipitated from cell lysates and analyzed by immunoblotting with two anti-ubiquitin MAbs. Both receptor isoforms displayed increased apparent molecular weight, when analyzed with the MAb P4D1 (Figure 5A), which recognizes mono-ubiquitin, as well as poly-ubiquitin chains. For OB-Ra, the molecular weight shift was limited. This observation is consistent with the conjugation of OB-Ra with a small number of ubiquitin moieties. For OB-Rb, a high molecular weight smear was observed up to the top of the blot. This is indicative of a larger number of ubiquitin conjugates. When analyzed with FK1, an MAb specific for poly-ubiquitin chains (Figure 5A), OB-Ra was not detected, and OB-Rb displayed a very weak signal at the top of the blot. These results suggest that OB-Ra and OB-Rb are primarily conjugated with mono-ubiquitin. Only a small fraction of the total expressed receptor appears to be ubiquitinated, since the ubiquitinated forms were not detected by immunoblotting without a prior concentration step by immunoprecipitation.

To confirm that the molecular weight shift of OB-Ra reflected ubiquitylation on lysine residues K877 and K889, OB-Ra, the single mutants 3KR and 2KRK, or the double-mutant 2K2R were co-expressed with His₆-tagged ubiquitin, by transient transfection. Ubiquitin-conjugates were purified on nickel beads, and OB-R constructs were detected by immunoblotting. For OB-Ra, 3KR, and 2KRK constructs, two immunoreactive bands were detected when His₆-tagged ubiquitin was co-expressed, whereas only the lower band was detected with the double mutant 2K2R (Figure 5B). The lower band could be detected in the absence of co-expressed His₆-tagged ubiquitin; it migrated to the same position as non-modified OB-Ra, and thus was tentatively identified as unconjugated OB-R, non-specifically pulled down with the beads. The higher band was only detected when OB-Ra or

Figure 4 Role of the lysine residues of the cytoplasmic domain in OB-Ra endocytosis. (A) Sequences of the cytoplasmic tails of OB-Ra and lysine-to-arginine mutants. The positions of lysine residues in the sequence of OB-Ra are indicated. (B) Internalization of OB-Ra mutants analyzed by antibody uptake. HeLa cells transiently expressing the indicated construct were allowed to internalize anti-HA MAb for 20 min and internalized antibody was detected by immunofluorescence. (C) Quantification of the impact of each mutation on endocytosis. The number of endosomes were counted in 120 randomly chosen cells transiently expressing each construct. In the graphic, the mean endosome number per cell is indicated as a measure of the relative internalization rate of each construct. Values not significantly different from each other by statistical analysis are indicated by a square bracket over the corresponding bars. (D) Internalization of OB-Ra mutants analyzed by cell surface biotinylation and glutathione cleavage resistance. The fraction of protein internalized during 15 min at 37°C was analyzed as in Figure 3C.

one of the single mutants were co-expressed with His₆-tagged ubiquitin, but not when OB-R constructs or His₆-tagged ubiquitin were expressed on their own. Thus, this band was representative of ubiquitin-conjugated OB-R species. No slower migrating bands or smears were detected in the upper part of the blots. The relative migrations of non-ubiquitylated and ubiquitylated receptor bands were similar for the wild type and mutant constructs, and were consistent with one OB-Ra polypeptide chain being conjugated with one or two ubiquitin molecules (Figure 5B). Taken together these data suggest that part of OB-Ra is modified on K877 and/or on K889, and that each lysine residue bears a limited number of ubiquitin molecules.

To determine if the ubiquitylation step occurs at the cell surface, OB-Ra endocytosis was blocked by dynamin-2 K44A overexpression, and ubiquitin-conjugated OB-Ra was quantified by immunoblotting. As expected for a membrane protein internalized by clathrin-mediated endocytosis, OB-Ra endocytosis was strongly inhibited by the overexpression of dynamin K44A (data not shown). Results (Figure 5C) show that the amounts of ubiquitylated OB-Ra were dramatically increased upon dynamin K44A overexpression, as compared with lysates from GFP-overexpressing control cells. This result suggests that OB-Ra is conjugated with ubiquitin at the cell surface (or before reaching the cell surface). This is consistent with a function for OB-Ra ubiquitin-conjugates in an early step of endocytosis.

The fusion of an ubiquitin molecule restores the endocytosis of an ubiquitylation-defective OB-Ra mutant

To test if mono-ubiquitin could function as an endocytosis signal, an ubiquitin molecule was fused at the C-terminus of the 4R mutant, which is both defective for endocytosis and for ubiquitylation. When expressed in HeLa cells, this new construct (4R-Ub) displayed a pattern of internalized antibody very similar to OB-Ra, in contrast to 4R, which was only detected at the plasma membrane (Figure 5D), indicating that the fusion of an ubiquitin molecule restored the endocytosis of the 4R mutant. Immunoblot analysis indicated that 4R-Ub was not further ubiquitylated by the cells (data not shown). The relative endocytosis rates of OB-Ra and 4R-Ub were quantified by cell surface biotinylation and glutathione resistance cleavage. The constitutively ubiquitylated 4R-Ub was internalized from the cell surface about two to three times faster than OB-Ra (Figure 5E), suggesting that ubiquitylation is a rate-limiting step of OB-Ra endocytosis.

The pattern of labeled endosomes was very similar for OB-Ra and 4R-Ub, suggesting that not only the initial internalization step, but also the intracellular transport up to the perinuclear endosomes were restored by the fusion of an ubiquitin molecule. In addition, 4R-Ub endocytosis was inhibited by CHC depletion (Figure 5F). These observations suggest that similar endocytosis routes were followed by OB-Ra and 4R-Ub. Taken together, the data confirm that an ubiquitin molecule functions as an endocytosis signal on OB-Ra, and imply that the ubiquitin-acceptor lysine residues K877 and K889 do not function as part of an ubiquitylation-independent endocytosis motif.

In order to verify if ubiquitin also functions as internalization motif for OB-Rb, all of the 12 lysine residues of OB-Rb cytosolic tail were changed for arginines by site-directed

mutagenesis. This lysine-less construct (OB-Rb12R) was expressed in HeLa cells and its endocytosis was examined by antibody uptake. Results shown in Figure 5D indicate that OB-Rb12R internalization was not inhibited. This suggests that another endocytosis motif is present in OB-Rb cytosolic tail.

Proteasome inhibitors block OB-Ra endocytosis by inhibiting its ubiquitylation

To study the molecular basis for the strong inhibiting action of proteasome inhibitors on OB-Ra endocytosis, we analyzed the effect of proteasome inhibitor treatment on OB-Ra ubiquitylation. OB-Ra was co-expressed with His₆-tagged ubiquitin and the cells were incubated for 2 h with lactacystin. Ubiquitin-conjugated proteins were purified on nickel beads and the presence of ubiquitylated OB-Ra was analyzed by immunoblotting. No ubiquitylated OB-Ra band was detected in the sample from lactacystin-treated cells, in contrast to control cells (Figure 6A). This result indicates that the pre-incubation of the cells with lactacystin resulted in the inhibition of ubiquitylation (or in the deubiquitylation) of OB-Ra. This blockade of OB-Ra ubiquitylation could provide a molecular explanation to the inhibition of OB-Ra endocytosis by proteasome inhibitors.

To examine the impact of proteasome inhibitor treatment on the levels of cellular conjugated and unconjugated ubiquitin, HeLa cells were transfected with HA-tagged ubiquitin, and incubated with lactacystin or chloroquine for 2 h. As expected, immunoblot analysis showed an accumulation of poly-ubiquitylated proteins in the upper part of the blot in lactacystin-treated cells (Figure 6B). In contrast, the intensity of the $\approx$ 8-kDa ubiquitin band, which represents the pool of unconjugated ubiquitin, was dramatically reduced after lactacystin treatment (Figure 6B). Chloroquine treatment, which inhibits OB-Ra degradation but not its internalization (Belouzard *et al*, 2004), did not significantly alter the levels of $\approx$ 8-kDa ubiquitin. This result suggests that the accumulation of poly-ubiquitylated proteins under proteasome inhibition partially depletes the cells from free ubiquitin molecules.

To determine if the inhibition of OB-Ra endocytosis by proteasome inhibitors resulted from the inhibition of ubiquitylation, or if additional effects of the preincubation with proteasome inhibitors could interfere with OB-Ra endocytosis, we compared the endocytosis of OB-Ra and of 4R-Ub (which is constitutively mono-ubiquitylated), in the presence of proteasome inhibitors. After 2 h of MG-132 treatment, the endocytosis of OB-Ra was totally blocked, whereas 4R-Ub was internalized in the presence of the drug as efficiently as in nontreated control cells (Figure 6C). Similar results were obtained with lactacystin (data not shown). Taken together, these results suggest that proteasome inhibitors interfere with the internalization step of OB-Ra endocytosis by blocking ubiquitin conjugation on lysine 877 and 889 residues, possibly by depleting the cells from free ubiquitin or by other mechanisms, and that they had no apparent additional inhibitory effects on the OB-Ra endocytosis pathway.

The fusion of an ubiquitin molecule targets an endocytosis-defective transferrin receptor mutant to a clathrin-mediated pathway of internalization

To determine if mono-ubiquitin could be a transferable endocytosis motif for clathrin-mediated endocytosis, we

fused an ubiquitin to the N-terminus of a HA-tagged transferrin receptor deleted from its own tyrosine-based endocytosis motif (TfRΔ3–59). This mono-ubiquitylated TfR construct was named Ub-TfRΔ3–59 (Figure 7A). To avoid the proteolytic cleavage of ubiquitin from the truncated TfR in Ub-TfRΔ3–59, the two C-terminal glycine residues of ubiquitin were deleted. The stability of the construct was verified by immunoblot (data not shown). As expected (Collawn *et al.*, 1993), the endocytosis of TfRΔ3–59 was dramatically reduced, as shown by the labeling of the plasma

membrane and the absence of endosomal staining (Figure 7B). This inhibition resulted from the deletion of the endocytosis motif centered on tyrosine 20, which targets TfR to clathrin-coated pits by interacting with the μ -chain of AP-2 adaptor complex (Hirst and Robinson, 1998). In contrast to TfRΔ3–59, both TfR and Ub-TfRΔ3–59 were detected in endosomes after an antibody uptake (Figure 7C). To assess the role of clathrin in their endocytosis, these two constructs were expressed in CHC siRNA-treated cells. As shown in Figure 7C, both TfR and Ub-TfRΔ3–59 were detected at the

Figure 6 Action of proteasome inhibitors on OB-Ra ubiquitylation. (A) Inhibition of OB-Ra ubiquitylation by lactacystin. The ubiquitylation of OB-Ra in the presence of lactacystin was analyzed as described in the legend of Figure 5. (B) Depletion of the cellular pool of ubiquitin by lactacystin. HeLa cells transfected with an expression vector for HA-tagged ubiquitin were incubated for 2 h in the presence of lactacystin or chloroquine. The cellular content of ubiquitin and ubiquitin conjugates was analyzed by immunoblotting with an anti-HA MAb. The band of unconjugated ubiquitin is indicated by an arrowhead. The upper part of the gel shows conjugated proteins. (C) Endocytosis of 4R-Ub in the presence of proteasome inhibitor. HeLa cells transiently expressing OB-Ra or 4R-Ub were incubated for 2 h in the presence or the absence of MG-132, as indicated. Endocytosis was analyzed as described in the legend of Figure 1.

Figure 5 Role of ubiquitylation in OB-Ra endocytosis. (A) Ubiquitylation of OB-Ra and OB-Rb. Lysates from HeLa cells expressing OB-Ra or OB-Rb or from control cells were immunoprecipitated with an anti-HA antibody and then immunoblotted with anti-ubiquitin MABs P4D1 or FK1. The migration of unconjugated receptors is indicated on the right side. Each membrane was reprobed with anti-HA MAB. The specificity of each MAB was assessed with an ubiquitin ladder. Note that P4D1, but not FK1, detects mono-ubiquitin. (B) Ubiquitylation of OB-Ra and lysine-to-arginine mutants. HA-tagged OB-Ra, 2R2K, 2K2R, 3KR, or 2KRK were expressed in HeLa cells, alone (–) or in combination with His₆-tagged ubiquitin (+). Ubiquitylated proteins were purified from cell lysates by pull-down on Ni-agarose beads, and the presence of OB-R was analyzed by immunoblotting. The migration of unconjugated and conjugated (bracket) OB-Ra is indicated on the left side. (C) Impact of dynamin 2 K44A overexpression on OB-Ra ubiquitylation. HA-tagged OB-Ra and/or His₆-tagged ubiquitin were expressed by transient transfection in HeLa cells and GFP-tagged dynamin 2 K44A was expressed with an adenoviral vector. Control cells were transduced with an equivalent dose of adenoviral vector expressing GFP. Ubiquitylated OB-Ra was purified on Ni-agarose beads and analyzed by immunoblotting. The migration of unconjugated and conjugated (bracket) OB-Ra is indicated on the left side. (D) Ubiquitin-dependent internalization of OB-Ra. Two new constructs were generated: an ubiquitin molecule was fused to the C-terminus of 4R (4R-Ub) and all lysine residues of OB-Rb were replaced with arginine (OB-Rb12R). OB-Ra, 4R, 4R-Ub, OB-Rb or OB-Rb12R were transiently expressed in HeLa cells and their internalization analyzed by antibody uptake. (E) Relative internalization rates of OB-Ra and 4R-Ub analyzed by cell surface biotinylation and glutathione cleavage resistance. The amounts of protein internalized at 37°C during the indicated time were analyzed as in Figure 3C, together with 20% of the biotinylated material (surface). (F) Clathrin-mediated endocytosis of 4R-Ub. Internalization of 4R-Ub was analyzed in cells treated with control or clathrin siRNA as described in the legend of Figure 1. As a control, the uptake of Alexa555-conjugated transferrin (Tf) was monitored in parallel.

plasma membrane after a 10-min uptake in cells treated with CHC siRNA. In contrast, TfR and Ub-TfRΔ3–59 were detected in endosomes in cells treated with control siRNA. These patterns are indicative of a clathrin-mediated internalization of TfR and of Ub-TfRΔ3–59. The levels of endocytosis inhibition imposed by CHC depletion were similar for both constructs (Figure 7D). Taken together, these data strongly suggest that mono-ubiquitin can function as a transferable internalization motif in clathrin-mediated endocytosis.

Discussion

Ubiquitin as an endocytosis motif

A number of cell surface receptors and membrane proteins have been reported to be ubiquitylated at the plasma membrane, and for some of them a role of the ubiquitylation in the downregulation has been demonstrated (Strous and Govers, 1999; Bonifacino and Traub, 2003; Hicke and Dunn, 2003; Marmor and Yarden, 2004). Yet the role of ubiquitin conjugates as endocytosis motif functioning in the initial internalization step at the plasma membrane has remained elusive in mammalian cells. In this article, we report that ubiquitin conjugates indeed function as endocytosis signal of a mammalian membrane protein, the leptin receptor isoform OB-Ra. We showed that a fraction of OB-Ra is ubiquitylated on two lysine residues of its cytoplasmic tail, that these two ubiquitin-acceptor residues are essential for internalization from the plasma membrane, and that the fusion of an ubiquitin molecule to the C-terminus of an ubiquitylation-defective mutant restores endocytosis. Consistent with a role of ubiquitin conjugates in OB-Ra internalization, the inhibition of endocytosis by dynamin K44A overexpression resulted in the accumulation of ubiquitylated OB-Ra. All these data strongly suggest that the ubiquitin molecules conjugated on lysine residues K877 and K889 of the cytosolic tail indeed function as endocytosis motifs.

Interestingly, we found that OB-Ra internalization depends on clathrin function. This suggests that ubiquitin conjugates can function as clathrin-dependent endocytosis motifs. This finding is quite different from two recent reports of membrane proteins that are internalized from the cell surface in an ubiquitin-dependent manner by a lipid raft-mediated, clathrin-independent pathway (Chen and De Camilli, 2005; Sigismund *et al.*, 2005). Unlike the above-mentioned studies, our data with 4R-Ub and Ub-TfRΔ3–59 suggest that an ubiquitin molecule fused to the cytosolic domain of a

Figure 7 Ubiquitin as a transferable motif in clathrin-mediated endocytosis. (A) Structure of the constructs. Tfr, HA-tagged human transferrin receptor; TfrΔ3–59, HA-tagged human transferrin receptor deleted from its cytoplasmic domain; Ub-TfrΔ3–59, HA-tagged human transferrin receptor, the cytoplasmic domain of which was replaced with ubiquitin. (B) Absence of internalization of TfrΔ3–59 monitored with a 20-min MAb uptake. (C) Clathrin-mediated internalization of Ub-TfrΔ3–59. HeLa cells were treated with control or clathrin siRNA and then transiently transfected with plasmid encoding Tfr or Ub-TfrΔ3–59. The cells were treated for 2 h with 50 μg/ml cycloheximid and incubated with anti-HA MAb for 10 min at 37°C, to label cell surface and internalized material. Anti-HA MAbs were detected with a cy3-conjugated secondary antibody after fixation and permeabilization. (D) Quantification of the internalization of Tfr and Ub-TfrΔ3–59 in control (white bar) or clathrin-depleted (gray bars) cells. Data are expressed as percentage of cells that internalized receptors.

membrane protein can function as a transferable internalization motif targeting to the clathrin-mediated pathway of endocytosis. The reason of this discrepancy is not clear at the present time. Our study was performed using transient transfection of expression vectors, because no cell line has been identified with high endogenous expression levels of leptin receptors. However, it is unlikely that our findings would only result from overexpression. Indeed, the use of immunofluorescence-based assays allowed us to examine individual cells with different expression levels, and we verified that the mechanisms of OB-Ra endocytosis were not dependent on the expression levels of the receptor.

Endocytosis motifs are thought to function by targeting to (or recruiting) specific budding machineries. By analogy with tyrosine- and dileucine-based motifs, which interact with AP-2 adaptor complexes, one can postulate the existence of specific adaptors able to bind mono-ubiquitin conjugates and recruit them to clathrin-coated pits. Epsin and EPS15, which are involved in endocytosis, are candidates. They bind clathrin and AP-2 and both have ubiquitin-binding domains (Benmerah *et al*, 1998; Rosenthal *et al*, 1999; Drake *et al*, 2000; Wendland, 2002). If they do function as adaptors in OB-Ra endocytosis, one could expect some redundancy in their function, as it was reported in yeast (Wendland, 2002). Ubiquitin-binding proteins, such as GGAs, Hrs, or TSG101 have been identified, which function in diverse parts of the endocytic and biosynthetic pathways to sort ubiquitylated membrane proteins (Bishop *et al*, 2002; Raiborg and Stenmark, 2002; Puertollano and Bonifacio, 2004; Scott

et al, 2004). These proteins also carry a clathrin-interacting domain and are localized in clathrin-coated domains of early endosomes and the TGN. It remains to be determined if some of them could also function at the plasma membrane, or if other proteins are specifically involved. OB-Ra is probably a good model to study the question, since its endocytosis only relies on ubiquitin motifs.

Inhibition of endocytosis by proteasome inhibitors

Our results strongly suggest an indirect action of proteasome inhibitors on OB-Ra endocytosis, as a consequence of the inhibition of their ubiquitylation. A similar inhibition of the ubiquitylation of HIV Gag precursor (Schubert *et al*, 2000), and of GHR (Strous and van Kerkhof, 2002) by proteasome inhibitors was previously reported. Although we did not thoroughly investigate the mechanism by which proteasome inhibitors block OB-Ra ubiquitylation, one possibility is that the inhibition of proteasome function, which reduces the cellular pool of free ubiquitin, results in levels of free ubiquitin low enough to interfere with OB-Ra ubiquitylation. Another possible mechanism could be the stabilization of a negative regulator of the ubiquitin ligase by proteasome inhibition. Such indirect actions would certainly explain why cells had to be treated for 2 h before the inhibition of OB-Ra internalization could be observed.

Consistent with this hypothesis of an indirect action of proteasome inhibition on OB-Ra ubiquitylation and endocytosis, the fusion of an ubiquitin molecule to the C-terminus of an ubiquitylation-defective mutant restored endocytosis even

in the presence of proteasome inhibitors. This indicates that the proteasome does not have any additional effect on OB-Ra internalization machinery. These findings are somewhat different from those reported for GHR. Like OB-Ra, GHR is ubiquitinated at the plasma membrane (Strous *et al*, 1996), and its endocytosis is inhibited by proteasome inhibitors (van Kerkhof *et al*, 2000). However, the mutation of all the lysine residues of its cytosolic tail impaired its ubiquitylation but not its endocytosis. Moreover, the endocytosis of this lysine-less GHR was still sensitive to proteasome inhibitors (van Kerkhof *et al*, 2000), suggesting that an accessory factor of the GHR endocytic machinery, rather than GHR itself, was regulated by an ubiquitylation step sensitive to proteasome inhibitors.

Endocytosis of leptin receptor OB-Rb

We found that OB-Rb is ubiquitinated, but that this ubiquitylation was not necessary for its internalization. This finding is quite similar to other membrane proteins that have been reported to be ubiquitinated at the plasma membrane (van Kerkhof *et al*, 2000; Marchese and Benovic, 2001; Rocca *et al*, 2001). Rather than acting as endocytosis motif, ubiquitin is involved in the sorting towards internal vesicles of compartments of the late endocytic pathway, which ultimately leads to degradation in lysosomes. Although proteasome inhibitors do not affect OB-Rb internalization, they do stabilize the protein. Stabilization of membrane proteins by proteasome inhibitors in late compartments of the endocytic pathway has been reported (Rocca *et al*, 2001; Bishop *et al*, 2002; Strous and van Kerkhof, 2002), but its molecular explanation is still missing. This was not further investigated for OB-Rb.

Although the function of OB-Rb ubiquitylation was not addressed in this study, it is probable that it is not essential for OB-Rb internalization from the cell surface, since lactacystin interferes with its degradation without effect on the initial internalization step, in contrast to OB-Ra. We cannot exclude that OB-Rb endocytosis may also be ubiquitin-dependent, but that its ubiquitylation may not have been fully abrogated by proteasome inhibitors. This hypothesis is however unlikely, since the lysine-less OB-Rb12R mutant was still internalized normally. This raises the possibility that other endocytosis motifs could be present in OB-Rb cytoplasmic domain, which would function in an ubiquitin-independent manner. Whether OB-Rb endocytosis also uses ubiquitin as endocytosis motif remains an open question.

Materials and methods

Cell Lines, DNA constructs, antibodies and chemicals

HeLa cells were maintained in minimal Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies).

Expression vectors for HA-tagged OB-Ra, OB-Rb and TfR were as previously described (Belouzard *et al*, 2004). Point mutations, deletions and ubiquitin fusions in OB-Ra, OB-Rb and TfR were generated by PCR-based mutagenesis, as previously described (Belouzard *et al*, 2004). All constructs were verified by DNA sequencing.

Constructs encoding N-terminal HA-tagged, or His₆-tagged ubiquitin expression vectors (Treier *et al*, 1994) were provided by Dirk Bohman (EMBL, Heidelberg, Germany). GFP-tagged Dynamin-2(aa) K44A and Rab5 expression vectors were provided by Mark A McNiven (Mayo Clinic, Rochester, Minnesota) and Marino Zerial (Max-Planck Institute, Dresden, Germany).

Polyclonal rabbit antiserum to Hrs was a generous gift from Harald Stenmark (The Norwegian Radium Hospital, Oslo, Norway).

Rat anti-HA MAb 3F10 was from Roche Molecular Biochemicals. Mouse anti-EEA-1 and anti-CHC MABs were from BD Biosciences. Goat polyclonal anti-actin and mouse anti-ubiquitin MAB (clone P4D1) were from Santa Cruz. Mouse anti-ubiquitin MAB (clone FK1) and polyubiquitin chains (Ub₂₋₇) were from Biomol International. Alexa555-conjugated human transferrin, and Alexa488-conjugated goat anti-mouse were from Molecular Probes. Cy3-conjugated goat anti-rat antibody was purchased from Jackson ImmunoResearch.

MG-132 was from Calbiochem. Other chemicals were from Sigma.

Immunofluorescence microscopy

HeLa cells grown on glass coverslips were transfected for 24 h using FuGENE-6 transfection reagent (Roche Molecular Biochemicals). To internalize anti-HA antibodies or Alexa555-conjugated transferrin, incubations were performed in serum-free medium. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Immunostaining was performed as previously described (Belouzard *et al*, 2004). Coverslips were mounted on slides with mowiol, and examined using an Axiophot 2 microscope (Zeiss) equipped with a cooled charge-coupled device Micromax (Princeton), or a Leica SP2 confocal microscope equipped with Ar/HeNe lasers and a PL APO 100x/1.40 oil immersion objective. Sequential excitations were carried out to exclude bleed-through effects. The images were merged using Adobe Photoshop.

RNA interference

Subconfluent cultures of HeLa cells in six-well plates were transfected twice with 80 pmol of synthetic double-stranded siRNA (Dharmacon) complexed with 4 µl of oligofectamine (Invitrogen) in a total volume of 1 ml of OptiMEM (Invitrogen) for 6 h. The interval between both siRNA transfections was 48 h. To assay the endocytosis, siRNA-treated cells were plated on glass coverslips, and transfected with expression plasmids using FuGene. CHC levels were analyzed by immunoblotting an equal amount of cell lysates using ECL (Amersham). The films were scanned and quantified with the image J software. The CHC target sequence was UAAUCCAAUUCGAGACCAAU (Motley *et al*, 2003). The control siRNA was originally designed to knock-down GFP, the target sequence is GCUGACCCUGAAGUUAUC.

Endocytosis assay by antibody uptake

HA-tagged OB-R constructs were expressed by transient transfection in HeLa cells. 10-min or 20-min antibody uptakes were carried out with the anti-HA MAB 3F10. Cells were quickly rinsed with PBS, fixed with 3% PFA, and the internalized antibody was revealed with an cy3-conjugated goat anti-rat IgG secondary antibody, after permeabilization with Triton X-100. Images from 30 randomly chosen, OB-R-expressing cells per cover slip were obtained with an Axiophot 2 microscope (Zeiss) equipped with a cooled charge-coupled device Micromax (Princeton). Endosomes were automatically counted using the Metamorph software, after processing the images with a median filter, thresholding and binarizing. Statistical analysis (analysis of variance and Student-Newman-Keuls' test) was performed using the SPSS software.

Endocytosis assay based on cell surface biotinylation

Cell surface biotinylation and resistance to the cleavage by reduced glutathione after internalization were as previously described (Belouzard *et al*, 2004).

Detection of ubiquitinated leptin receptors

HeLa cells grown on 10 cm dishes were transiently co-transfected using FuGENE-6 transfection reagent with plasmid DNA encoding His₆-tagged ubiquitin and HA-tagged OB-R constructs. GFP-tagged dynamin-2 K44A (or GFP in control cells) was expressed with a recombinant adenoviral vector. Detection of ubiquitinated OB-R was as previously described (Treier *et al*, 1994). Briefly, His₆-tagged ubiquitin conjugates were purified on Ni-agarose beads (QiaGene), and ubiquitinated OB-Rs were detected by immunoblotting with anti-HA MAB 3F10.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Note added in proof

While this manuscript was under revision, Geetha *et al* (*Mol Cell* 2005 **20**: 301–12) published that ubiquitylation of the nerve growth factor receptor TrkA mediates its endocytosis.

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