Ubc13 and COOH Terminus of Hsp70-interacting Protein (CHIP) Are Required for Growth Hormone Receptor Endocytosis*

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Background: The scientific question that we address is how cytokine receptors organize their degradation.
Results: CHIP and Ubc13 are required for GH receptor endocytosis, implicating Lys63-specific ubiquitination.
Conclusion: This study shows how two ubiquitin ligases act in concert to allow receptor endocytosis.
Significance: Understanding this mechanism enables drug design to control GH signaling in fighting cancer and cachexia.

Growth hormone receptor (GHR) endocytosis is a highly regulated process that depends on the binding and activity of the multimeric ubiquitin ligase, SCFβTrCP (Skp Cullin F-box). Despite a specific interaction between β-transducin repeat-containing protein (βTrCP) and the GHR, and a strict requirement for ubiquitination activity, the receptor is not an obligatory target for SCFβTrCP-directed Lys48 polyubiquitination. We now show that also Lys63-linked ubiquitin chain formation is required for GHR endocytosis. We identified both the ubiquitin-conjugating enzyme Ubc13 and the ubiquitin ligase COOH terminus of Hsp70 interacting protein (CHIP) as being connected to this process. Ubc13 activity and its interaction with CHIP precede endocytosis of GHR. In addition to βTrCP, CHIP interacts specifically with the cytosolic tails of the dimeric GHR, identifying both Ubc13 and CHIP as novel factors in the regulation of cell surface availability of GHR.

Growth hormone receptor (GHR) functions in longitudinal growth and metabolism. It is a prototypic member of the class I cytokine receptor family that, after binding to its natural ligand, growth hormone (GH), signals via Janus kinase 2 (Jak2). GH binding to the dimeric GHR induces a rotation in the GHR tail (1, 2) that subsequently leads to activation of Jak2 and, ultimately, to activation of signaling cascades via Stat5b, ERK, and MAP kinase pathways (reviewed in Ref. 3). Endocytosis and degradation of the GHR determine largely the sensitivity of cells for GH (4). GHR is endocytosed via clathrin-coated pits and transported via endosomes toward the lysosomes. To respond to different environmental signals, cells use specific tools to keep their responsiveness for growth factors and nutrients at the appropriate level. For many signaling receptors, entry into clathrin-coated pits depends on an active ubiquitination system. Although GHR ubiquitination is not required, GHR depends on the F-box protein β-transducing repeat-containing protein (βTrCP) for endocytosis (5). βTrCP binds to the ubiquitin-dependent endocytosis (Ube) motif of the GHR as part of the ubiquitin ligase complex SCFβTrCP (Skp1, Cullins, F-box proteins) (6). The same ligase is required to transport the GHR from endosomes to lysosomes (7).

There is emerging evidence for the involvement of Lys63-linked ubiquitination in the homeostasis of specific membrane proteins. In many instances, Ubc13 acts as the E2 enzyme that, together with pseudo E2s, UEV1A, or MMS2, assembles Lys63-linked ubiquitin chains on the substrates (8, 9). The dopamine transporter is provided with four-ubiquitin Lys63-linked chains, presumably via Nedd4-2 (10). UEV-1/Ubc13 is implicated in the regulation of an AMPA-type glutamate receptor in Caenorhabditis elegans (11). The yeast monocarboxylate transporter, Jen1, requires HECT-ubiquitin ligase Rsp5-dependent Lys63 ubiquitination for endocytosis (12). Short chain Lys63 ubiquitination mediates the regulated endocytosis of the aquaporin-2 water channel (13), and forced expression of ubiquitin mutants indicates that Lys63 ubiquitination of the prolactin receptor is important for its degradation (14). Chain assembly on substrates is an orchestrated interplay between an ubiquitin activating enzyme (E1), an conjugating enzyme (E2), and an ubiquitin ligase (E3). As exemplified, Ubc13/UEV1A utilizes several ubiquitin ligases to specifically ubiquitinate the substrates. In this study, we identified the COOH terminus of Hsp70 interacting protein (CHIP) as a specific E3 for GHR endocytosis. CHIP is a 35-kDa multi-domain protein containing an NH2-terminal tetratricopeptide repeat (TPR) and a COOH-terminal U-box domain. The U-box is related to the RING domain and acts passively as a scaffold for the E2, positioning it in proximity to the substrate. CHIP can act together with either UbcH5a or Ubc13/UEV1a to assemble either Lys48-
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or Lys63-linked chains, respectively. In both cases, the interaction is between the U-box and the conserved SPA motif of the E2 enzymes (15–17). CHIP binds with its TPR domain to the COOH-terminal EVD sequence of the molecular chaperones Hsp70 and Hsp90 (18). Interaction of CHIP with the two E2s, UbcH5a and Ubc13, has distinct effects on the conformational dynamics of CHIP, suggesting different roles of the CHIP-E2 interaction in the ubiquitination of substrates (19). CHIP links the Hsp70/Hsp90 protein quality control/folding machinery with the ubiquitination/proteasomal degradation pathways, making it a fate-deciding point for proteins. In addition, functions independent of Hsp70 and Hsp90 have also been reported in glycoprotein quality control (with SCF^{E6AP}), in the degradation of the Notch signaling factor Tal1 (with SCFSkp2), in controlling cellular levels of base excision repair enzymes, in the degradation of toxic forms of α-synuclein, and in the regulation of Smad1/5 proteins (20–25).

In this study, we describe a specific role of Lys63-linked ubiquitin chains and the E2/E3 pair Ubc13/CHIP in GHR endocytosis. Combining gene silencing and overexpressing approaches, the roles of the CHIP TPR domain, as well as the Ubc13 SPA motif in GHR endocytosis, were demonstrated. The GHR specificity is controlled by sequence information within and downstream of the UbE motif. We propose that the CHIP-Ubc13 activity occurs after the SCF^{EVTCP} ubiquitin ligation activity and before GHR selection into clathrin-coated pits.

EXPERIMENTAL PROCEDURES

Materials, Antibodies, DNA Constructs, and Cell Lines—Antibody anti-GHR (T) was raised in rabbits against the cytoplasmic sequence between amino acids 271 and 381, as described previously (26). Anti-CHIP antibody was obtained from Calbiochem, monoclonal anti-HA antibody was from Babco (Richmond, CA), Cy3-GH was prepared as described previously (6), Alexa 488-transferrin was from Molecular Probes, and EGFR-Alexa Fluor 488 streptavidin was from Invitrogen. DNA constructs CHIP and CHIPΔTPR were gifts from Dr. Douglas Cyr (University of North Carolina, Chapel Hill, NC), and Ubc13 and Ubc13 C78A in pEF-IREs-puro were gifts from Dr. James Chen (Southwestern University, Dallas, TX). The A98D mutation in Ubc13 was introduced using a QuikChange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The primers used for this reaction were 5’-TTGAAAAGATTAAGGTGCCCGATCTCCGATCCGCACAGTTCTG-3’ and 5’-CAGAAGCTTGGCAGATCTGGAGATCTGGAACCGACCATTATCTTTACA-3’. GST constructs were described previously (27). Dr. Matthias Mayer (Universität Heidelberg) kindly provided the CHIP overproducing strain (F18202 transformed with pUHE21–2idΔ12-hCHIP). U2OS cells expressing either GHR or both GHR and EGFR were described previously (7).

Cell Culture—U2OS cells were grown in DMEM (Invitrogen) supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 12 μg/ml Blasticidin S (MP Biomedicals). All of the cells were grown at 37 °C with 5% CO2. The cells were washed with PBS, detached from the flask with Trypsin-EDTA (Invitrogen), diluted in fresh growth medium, and split into new culture flasks twice a week.

Transfections—The cells were transfected with 40 nM validated small interfering RNAs specific for Ubc13 (5’-AATGGCGAGCCCCTAAGTGACG-3’), CHIP (STUB1; Ambion 289568) or Control#1 (Ambion AM4635) using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. DNA transfections were performed using FuGENE 6 (Roche Applied Sciences) according to the instructions of the manufacturer.

Immunofluorescence Microscopy—GHR- or EGFR-expressing U2OS cells were grown on coverslips and allowed to take up Cy3-GH and/or Alexa 488-EGF at 37 °C for 30 min. The cells were washed and fixed with 3% paraformaldehyde. The cells were permeabilized with 0.2% Triton X-100 for 5 min and washed three times. The fixation was terminated in 0.5% BSA in PBS for 15 min. The coverslips were incubated with the primary antibody in 0.5% BSA in PBS for 30 min. After washing with 0.5% BSA in PBS, the cells were incubated with secondary antibody for 30 min or directly mounted using Prolong Gold DAPI and analyzed using a Zeiss LSM510 confocal microscope and Zen Software.

125I-GH Uptake—125I-Labeled human GH was prepared with the use of chloramine T (26), and iodinated GH was purified over a 500-μl Zeba spin column (Pierce Thermoscientific). For internalization studies, the cells were grown in 12-well plates, treated with siRNAs as described above; washed with DMEM; supplemented with 20 mM, Hepes, pH 7.4, and 0.1% BSA; and incubated in a water bath. 125I-GH (180 ng/ml) was bound on ice for 2 h. Subsequently, the cells were washed free of unbound ligand and incubated at 37 °C for 0–15 min. Membrane-associated ligand was removed by acid wash (0.15 m NaCl, 50 mM glycine, 0.1% BSA, pH 2.5) on ice. Internalized ligand was determined by measuring the radioactivity after solubilization of the acid-treated cells in 1 n NaOH in a LKB γ-counter.

Biotin-GH Pulldown and Immunoprecipitations—Cells were lysed with lysis buffer (1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% BSA in PBS) on ice for 20 min. The lysates were clarified for 5 min, and the supernatants were incubated with biotin-GH or anti-GHR (T) for 1 and 2 h, respectively, followed by 1 h of incubation with streptavidin-agarose or protein A-Sepharose. Alternatively, for pulling the GHRs at the cell surface, HEK293-TR cells were placed on ice, and the culture medium was replaced with cold DMEM (20 mM Hepes, pH7.4), after which biotin-GH (180 ng/ml) was added. After 2 h of incubation, which results in saturating GHR binding to biotin-GH, the cells were washed three times with PBS, and lysed with lysis buffer. After 5 min of centrifugation, the supernatant was incubated for 1 h with streptavidin-agarose beads. The beads were washed three times with lysis buffer and three times with PBS. The samples were subjected to reducing SDS-PAGE and transferred to Immobilon-FL polyvinylidenedifluoride membrane (Millipore).
Detection and quantification of the signals were performed with an Odyssey system (LI-COR Biosciences).

For ubiquitination experiments, the cells were lysed in 1% Triton X-100 with inhibitors (1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM N-ethylmaleimide, 1 mM Na₂VO₄, and 100 mM NaF). The cell lysates were centrifuged at maximum speed in an Eppendorf centrifuge for 30 min at 4 °C, and the supernatants were used for GHR isolation with anti-GHR antibody (anti-T) and protein A beads in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% BSA, and inhibitors. Immunoprecipitates were subjected to reducing SDS-PAGE and Western blotting.

Recombinant CHIP and βTrCP Purification—Induction of CHIP expression was performed in 1.5-liter cultures of the bacterial host FI8202, at A₆₀₀ = 0.8 with 250 μM isopropyl-1-thio-β-D-galactopyranoside, at 30 °C for 3 h. After harvesting the culture, the pellet was resuspended in 50 ml of buffer A (50 mM Hepes, pH 7.0, 50 mM KCl, 5 mM DTT, 10% glycerol, 1 mM PMSF), supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin) and 0.4 mg/ml lysozyme. The suspension was left on ice for 30 min, after which the cells were lysed by sonication. After removing the cell debris by centrifugation at 20,000 × g for 20 min, the supernatant was subjected to ultracentrifugation at 100,000 × g for 2 h. The supernatant was recovered and precipitated with 40% ammonium sulfate. After centrifugation at 10,000 × g for 30 min, the pellets were resuspended in 10 ml of buffer A and dialyzed against the same buffer overnight. The samples were subjected to ultracentrifugation at 100,000 × g for 1 h, and the filtered supernatant was loaded onto an anionic exchange column (DEAE FF16/10 1×20 ml). The column was washed with 1 column volume of buffer A, and bound proteins were eluted with a linear gradient of 0 to 100% buffer B (50 mM Hepes, pH 7.0, 1 mM KCl, 5 mM DTT, 10% glycerol, 1 mM PMSF) over 5 column volumes. CHIP-containing fractions were pooled, concentrated to 500 μl, and loaded on a S200 gel filtration column pre-equilibrated with 2 column volumes of buffer A. The gel filtration step resulted in three distinct peaks, corresponding to different molecular weight CHIP forms. A mixture of three forms was used in the experiments. βTrCP purification from insect cells is described elsewhere.⁴

GST Fusion Protein Production and Pulldown Competition Experiments—GST fusion proteins were produced and purified according to the GST fusion protocol (Qiagen). The plasmids were transfected in a BL21 Rosetta strain and grown at 37 °C. At A₆₀₀ = 0.5, the expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 30 °C. GST fusion proteins were purified from bacterial lysates with glutathione-Sepharose beads for 2 h. The beads were washed six times with lysis buffer and then added to either cell lysates containing CHIP, purified CHIP, or βTrCP in lysis buffer and incubated for 2 h. The beads were washed three times with lysis buffer and three times with PBS. For the competition experiments, the beads were additionally incubated for 2 h with the indicated concentration of competing protein and washed again. The beads were boiled in SDS sample buffer and analyzed by Western blotting. The proteins were separated using SDS-PAGE and transferred to blot by Western blotting. The blots were detected using indicated antibodies with an Odyssey system.

RESULTS

Lys⁶³-linked Ubiquitin Chains Are Involved in GHR Endocytosis—To investigate the role of Lys⁶³-linked ubiquitin chains in GHR endocytosis, we expressed wild type and mutant K63R ubiquitin, unable to form Lys⁶³-linked chains, in GHR-expressing U2OS cells. To monitor endocytosis, we incubated cells with Cy3-GH for 30 min. In cells overexpressing HA-ubiquitin K63R, we observed a clear increased cell surface labeling of Cy3-GH, compared with wild type ubiquitin, indicative of inhibition of GH degradation (Fig. 1). Because not all cells synthesized equal amounts of GHR, some HA-positive cells show a moderate response because of very low GH-binding sites. This finding indicates that Lys⁶³-linked ubiquitin chains are involved in GHR degradation.

GHR Endocytosis Requires Both Ubc13 and CHIP—Because GHR endocytosis was Lys⁶³-dependent GHR degradation, we asked whether Ubc13 is required for this process. In complex with the pseudo E2 enzymes UEV1 or MMS2, Ubc13 can assemble Lys⁶³-linked ubiquitin chains (8). We assessed the uptake of Cy3-GH by confocal fluorescence microscopy in cells silenced for Ubc13. Fig. 2A shows a strong accumulation of Cy3-GH signal on the cell surface of cells that were treated with Ubc13-specific siRNA that did not occur in cells treated with control siRNA.

To identify the E3 that is involved in this process, we transfected the cells with five siRNAs to silence the expression of Triad1, CHIP, CHFR, Parkin, and RNF85, known to interact with Ubc13. As seen in Fig. 2A (right panels), transfection of siRNA, specific for the ubiquitin ligase CHIP, revealed similar accumulation of Cy3-GH as in Ubc13-depleted cells, indicating that Ubc13 might function together with CHIP in the GHR

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**FIGURE 2.** GHR endocytosis requires Ubc13 and CHIP. A, U2OS cells expressing GHR were treated with Ubc13, CHIP, or control siRNA for 3 days; incubated with Cy3-GH and Alexa 488-Tf for 30 min; and fixed. Localization of Cy3-GH and transferrin was determined with confocal microscopy. Bars, 20 μm. B, the cells were treated with CHIP or control siRNA for 2 days. The cell lysates were boiled in sample buffer and analyzed using Western blot and immunostaining with anti-GHR(B), anti-CHIP, and anti-actin. C, cells were labeled for 15 min in the presence of [35S]methionine and then chased for 15 min. After lysis, GHR was immunoprecipitated using anti-GHR(B) and analyzed on a Storm imaging system after electrophoresis. TCL, total cell lysate. D, after silencing for 3 days, initial endocytosis rates were measured 5, 10, or 15 min after a 2-h incubation with 180 ng/ml [125I]-GH on ice. The amount of intracellular radioactivity was measured and expressed as a percentage of total radioactivity. All of the data in this figure are representative of four independent experiments. *, p < 0.001 compared with control.

The results presented above show that depletion of either Ubc13 or CHIP inhibits GH endocytosis, as well as GHR degradation. To confirm that this is due to defective ubiquitin conjugation, we investigated the effect of mutations in critical residues of Ubc13 on GHR endocytosis. Cells contain sufficient Ubc13 activity to serve ongoing Lys63-linked ubiquitination: (over)expression of exogenous wild type Ubc13 does not induce phenotypic effects. However, if the active cysteine residue of Ubc13 is mutated to alanine (C87A), the conjugation activity is no longer functional, and expression of such a mutant acts as dominant negative for Ubc13-driven ubiquitination. As seen in Fig. 3A, exogenous expression of Ubc13(C87A) resulted in a clear inhibition of Cy3-GH uptake, whereas exogenous wild type Ubc13 expression did not interfere. This shows that enzyme activity of Ubc13 is essential for proper GHR endocytosis. The dominant negative effect indicates that Ubc13 C87A competes with endogenous Ubc13 for the binding to a ubiquitin ligase. Ubc13 interacts with CHIP through its SPA motif (15). To test whether the SPA motif on Ubc13 is essential for the dominant negative effect on GHR endocytosis, we introduced the A98D mutation into the Ubc13(C87A) mutant, disrupting Ubc13-CHIP binding. As expected, the dominant negative effect was abolished, and the cells endocytose Cy3-GH compa-

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rably with cells transfected with either wild type or Ubc13(A98D). Based on our screen results, showing that no other the Lys63-specific E3 showed a GHR-specific effect on Cy3-GH endocytosis, we conclude that both Ubc13 activity and the CHIP-Ubc13 interaction are needed for proper GHR endocytosis.

CHIP Silencing Does Not Inhibit General Clathrin-mediated Endocytosis—Because ubiquitination by CHIP has important general functions in protein homeostasis and chaperone activities in connection with Hsp70/90, it is important to ascertain that its action in GHR endocytosis is specific, rather than part of the general clathrin-mediated endocytosis machinery. In Fig. 2, we showed that transferrin receptor endocytosis requires neither CHIP nor Ubc13. To investigate whether CHIP is also involved in the endocytosis of the EGF receptor, prototypic for many receptor tyrosine kinases (37), we used U2OS cells expressing both GHR and EGF receptor. Fig. 3B shows that Alexa 488-EGF uptake is not affected in cells depleted for CHIP. Together with the observations for transferrin (Fig. 2), this implies that CHIP affects neither endocytosis of a prototypic receptor tyrosine kinase (EGF receptor) nor the endocytosis of transferrin.

CHIP Interacts with GHR—To unravel the role of CHIP in GHR endocytosis further, we analyzed the CHIP-GHR binding. We transiently expressed Myc-tagged CHIP in GHR-expressing U2OS cells and addressed the GHR-CHIP interaction using both biotin-GH and anti-GHR immunoprecipitation (Fig. 4A). Alternatively, we co-expressed GHR and Myc-CHIP in HEK293-TR cells and isolated the complexes in the same way (Fig. 4B). In both cases, specific interaction of Myc-labeled

FIGURE 3. Ubc13 activity is essential for GHR endocytosis. A, GHR-expressing U2OS cells were transfected with HA-tagged constructs of Ubc13 or Ubc13 mutants (C87A, A98D, C87A, and A98D) and incubated with Cy3-GH for 30 min at 37 °C. The cells were fixed and immune stained with anti-HA and a secondary antibody conjugated with Alexa 488. Localization of Cy3-GH and HA was determined by confocal microscopy. B, CHIP acts specifically on GHR endocytosis. U2OS cells expression both GHR and EGF receptor were transfected with siRNA specific for CHIP or control siRNA and incubated with Cy3-GH and Alexa 488 EGF for 30 min. The cells were fixed, and the labels were visualized on a LSM510 confocal microscope using Zen software. Bar, 20 μm. The data in this figure are representative of three independent experiments.
CHIP with GHR was detected, despite some background binding of CHIP to the beads in the absence of expressed GHR. We conclude that CHIP specifically binds to GHR. An important question is whether CHIP indeed interacts with GHR at the cell surface. To address this, we co-expressed GHR and Myc-CHIP in HEK293-TR cells and incubated the cells with biotin-GH on ice. Fig. 4C shows that CHIP specifically interacts with the GHR at the cell surface.

Previously, we reported the small glutamine-rich TPR-containing protein (SGT) to bind via its first TPR to the UbE motif of the GHR (27), but gene silencing of SGT did not result in an endocytosis phenotype. To examine whether the CHIP-GHR interaction involves the TPR domain, we used CHIP lacking the TPR domain. Using biotin-GH, we were able to pull down Myc-CHIP, but not Myc-CHIPΔTPR, together with full-length GHR, indicating that indeed the protein-protein interacting TPR domain is involved in binding CHIP to GHR (Fig. 4D). Attempts to detect binding between Ubc13 and GHR failed, probably because of the transient character of the interaction. Next, we investigated whether CHIP, present in total cell lysates, was able to bind purified GHR cytoplasmic tails. Using identical GST fusion proteins as in the SGT study, Fig. 4E shows specific interaction that depends on the presence of amino acid residues between 318 and 334 in the GST fusion protein, confirming that the binding is specific (27).

CHIP Binds GHR Independently of βTrCP—CHIP recognizes the acidic (M/I)EEVD consensus sequence in Hsp90 and Hsp70 (18). Additionally, CHIP can bind to exposed hydrophobic parts of unfolded proteins (38). The results from Fig. 4E suggest that the sequence between residues 318 and 334 is important for CHIP-GHR binding. We noticed that the GHR tail, between amino acids 320 and 350, contains an acidic-rich region that clearly extends beyond amino acid residue 334, the COOH-terminal amino acid residue of our GST fusion protein used in the Fig. 4. To ascertain that we test the GHR-CHIP interactions to the full extent, we used a longer GST-GHR fusion protein, ranging from amino acids 270 to 375, and untagged CHIP, produced in Escherichia coli. Using this assay, we addressed two questions: 1) Is the CHIP-GHR interaction indeed direct and saturable? 2) How does CHIP interact with GHR in the presence of SCFβTrCP, the other E3, involved in GHR endocytosis? As seen in Fig. 5A, both recombinant βTrCP and CHIP bound the GST-GHR270–375 in a concentration-dependent and saturable manner. The previously described UbE motif (321DDSWVEFIELDIDE334) was found to be involved in βTrCP binding (6). To address the second question and to investigate whether βTrCP and CHIP bind independently or cooperatively to the same region of GHR, we performed competition experiments. Beads coupled to GST-GHR270–375 were saturated with either CHIP or βTrCP (purified from SF9 cells) and incubated with increasing amounts of the “competing” interactor. We observed that CHIP binding to GHR could not be competed off by βTrCP, and vice versa. Additionally, we assessed the binding of CHIP to GST-GHR270–375 in the presence and absence of βTrCP. Fig. 5B shows no difference in CHIP binding. We conclude from these data that CHIP and βTrCP do not compete for binding on the GHR tails used and that no cooperativity exists between βTrCP and CHIP for binding to the (monomeric) GHR tails. This implies the existence of two different, independent binding sites for βTrCP and CHIP.

Ubc13 and CHIP Act after βTrCP during GHR Endocytosis—Once it is established that both βTrCP and CHIP are required...

5 P. van Kerkhof, personal communication.
for GHR endocytosis and that both bind to the cytosolic tail segment 318–375, it is important to determine their respective roles. Most probably both are required at a stage before GHR recruitment into clathrin-coated pits. Because both factors act in GH-dependent as well as independent (constitutive) endocytosis, there is no easy parameter to measure the progression toward recruitment into coated pits. Therefore, we used the ubiquitination state of GHR as a tool to assess the order of events in GHR endocytosis. As previously shown, TrCP depletion abolishes GHR (Lys48) ubiquitination, indicating that GHR is a substrate for TrCP. We also showed that depletion of clathrin not only inhibits GHR endocytosis but also causes a strong accumulation of ubiquitinated GHR at the cell surface (39). Taking GHR ubiquitination as a measure of the activity of SCF TrCP, we tested the effect of both Ubc13 and CHIP depletion on the GHR ubiquitination. As seen in Fig. 6, depletion of Ubc13 caused a significant accumulation of ubiquitinated GHR, which is comparable with the accumulation seen upon clathrin silencing. CHIP silencing resulted in a modest increase in the amount of ubiquitinated GHR, but this accumulation was not statistically significant when compared with the control situation. Overall, these results suggest that the pair Ubc13/CHIP acts downstream of SCF TrCP. These results are in line with our previous observation that K63R overexpression has little effect on the ubiquitination state of GHR (39). The results with anti-GHR (Fig. 6) show that gene silencing of neither Ubc13 nor CHIP increased the (130 kDa) GHR protein levels in contrast to the effects of silencing clathrin and TrCP. This is possibly due to a shift of GHRs to higher molecular weight (ubiquitinated) species that are very difficult to quantify. Together, we conclude that, although CHIP and TrCP can bind GHR independently, they act consecutively.

**DISCUSSION**

We have shown that both TrCP and Ubc13/CHIP are required for GHR endocytosis. Although their binding sites partly overlap, they bind independently. Although the binding of TrCP is strictly confined to the UbE motif and is defined as unconventional compared with the conventional DSGXXS motif present in β-catenin and IκB,4 the CHIP-GHR interaction extends beyond the UbE motif. Our results show that GHR accommodates two completely different E3 enzymes, both required for its endocytosis, involved in different ubiquitination activities. Because GHR with all lysine residues replaced by arginines is endocytosed as wild type GHR (5), we assume that both E3s target other substrates than GHR itself.
The GHR-CHIP interaction depends on the presence of a TPR domain in CHIP. This domain consists of three copies of the helix-turn-helix TPR motif that, together with a seventh COOH-terminal helix, functions in many signaling protein complexes. NMR and CD spectroscopic studies have revealed that this domain is largely unfolded until it interacts with a MEEVD pentapeptide derived from Hsp90 (38) that results in a stably folded structure. Because of the high content of acidic amino acid residues in the presumed binding region of the GHR between amino acids 320 and 350 (three sets of di-acidic conserved sequences), it is tempting to assume that they are part of a CHIP-binding site, analogous to the MEEVD sequence in Hsp90. Binding experiments with an array of alanine point-mutated GST-GHR peptides did not reveal a CHIP-specific binding motif between amino acid residues 320 and 350 (not shown). This might be because mutating single amino acids is not sufficient to considerably weaken the interaction or because of a temperature-sensitive complex (38, 40). Because both βTrCP and CHIP can bind to the same extent without mutual interference, a possible binding site for CHIP would be the amino acid sequence IDDPDE (rabbit) or IDEPDE (human GHR), located immediately after the UbE motif. Our experiments show that CHIP binds both to (dimeric) GHR isolated from cells as to (monomeric) GST fusion proteins. As we performed the competition studies in the latter system, the results suggest that both E3s can bind to the same GHR polypeptide.

There are indications for a role of ubiquitination in the endocytosis machinery. This is best illustrated by the necessity of ubiquitin-binding domains (UBDs) in the endocytosis adaptors Epsin and Eps15 that can also act as scaffold proteins (41). Because both adaptors can also be monoubiquitinated, it has been proposed that they can be in a closed, inactive conformation if the UBD binds to the attached monoubiquitin moiety (42, 43). In the open configuration, they can bind to ubiquitinated cargo via the UBD (44). The role of ubiquitin in endocytosis remains unclear: on the one hand, cargo-specific ubiquitination may be required to enable binding to the UBD of an adaptor; on the other hand, monoubiquitination of the adaptor (Eps15, Epsin) may be inhibitory because of its closed conformation. Because CHIP ubiquitinates Epsin, it is probably a negative factor in the endocytosis of cargo that needs Epsin for endocytosis such as in the case of Notch and the EGF receptor (45–47). In this study, CHIP is clearly acting as a positive factor for GHR endocytosis.

The role of ubiquitination in endocytosis of specific membrane proteins is slowly emerging as recently reviewed in Ref. 48. For some membrane proteins, cargo-specific ubiquitination has been reported. The epithelial sodium channel requires the ubiquitination machinery directed via Nedd4-2 (49). The IGF-I receptor is ubiquitinated by both Mdm2 (Lys63) and c-Cbl.
chains are required for sorting in endosomes via the ESCRT
complex, but there are few indications for their involvement in
endocytosis (48, 52). In yeast, the monocarboxylate transporter,
Jen1, requires Rsp5-dependent Lys63 ubiquitination for endo-
cytosis (12). In mammalian cells, the Kaposi’s sarcoma-associ-
ated herpesvirus-encoded ubiquitin E3 ligase, K3, ubiquitinates
cell surface MHC class I molecules, causing the internalization
and degradation of MHC I. In this process, K3 recruits the cel-
lar Ubc13 to generate Lys63-linked polyubiquitin chains on
MHC I, leading to its clathrin-mediated endocytosis. Our study
is the first that reveals a cellular machinery that requires Lys63-
linked ubiquitination in cargo selection at the cell surface. Pre-
viously, we demonstrated that SCFβTrCP is indispensable for
GHR to pass the endosomes (7). Because CHIP and SCFβTrCP
might act together at the cell surface, they might also do so in
endosomes. Actually, Fig. 1 shows Cy3-GH accumulation, not
only at the cell surface, but also intracellularly, if Ub K63R is
overexpressed.

The likelihood that CHIP and Ubc13 act together in GHR
endocytosis is strengthened by the experiments with overex-
pression of Ubc13 mutants, together with the observation that
overexpression of K63R ubiquitin blocks GHR endocytosis.
Insight in the conformational dynamics of CHIP with E2
enzymes and substrates other than chaperones indicate that
Lys63 ubiquitination via CHIP regulate processes in membrane
trafficking (53), and DNA repair (54). In contrast to CHIP in
complex with UbcH5a, CHIP in complex with Ubc13 can bind
to chaperones via the TPR domain, indicating that binding to a
substrate like GHR through the TPR domain does not interfere
with ability of CHIP to synthesize Lys63-linked ubiquitin
chains. Another question in this regard is our previous obser-
vation that GHR, truncated at amino acid 334, requires
SCFβTrCP for endocytosis (6). Because the binding site of CHIP
extends downstream of amino acid 334, it might mean that
removing this binding site would also eliminate the necessity of
CHIP. This is reminiscent of the involvement of the protea-
some: its action is required for the endocytosis of the full-length
receptor but is dispensable for a receptor truncated after amino
acid amino acid 366 (55). However, the GHR truncated at 334
might still have sufficient binding affinity for CHIP to act as a
necessary factor for endocytosis of the GHR334.

On the cooperativity of CHIP with other E3s, two action
modes have been proposed: 1) in neurons, CHIP acts with Fbx2
in glycoprotein quality control, where an NH2-terminal PEST
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dependent growth hormone receptor dimerization occurs in the endo-

Ubc13 and CHIP Involved in GHR Endocytosis

(0.00005). The prolactin receptor requires Lys63 polyubiquiti-

nation for endocytosis (14), as well as aquaporin-2 (13). Endo-
cytosis of the EGF receptor is accommodated by c-Cbl, although discussion about the necessity of covalent ubiquitin
attachment remains (51). In fact, except for the epithelial
sodium channel, strict proof of the necessity of covalent attach-

ment to the cytosolic tail of mammalian cargo molecules has
not been established. For GHR endocytosis, ubiquitination is a
major requirement, although the ubiquitin chains do not need
to be covalently attached to the receptor. Remarkably, this is the
case for both E3s described in this study.

In mammalian cells and in yeast, Lys63-linked ubiquitin
chains are required for sorting in endosomes via the ESCRT
complex, but there are few indications for their involvement in
downstream of amino acid 334, it might mean that
removing this binding site would also eliminate the necessity of
CHIP. This is reminiscent of the involvement of the protea-
some: its action is required for the endocytosis of the full-length
receptor but is dispensable for a receptor truncated after amino
acid amino acid 366 (55). However, the GHR truncated at 334
might still have sufficient binding affinity for CHIP to act as a
necessary factor for endocytosis of the GHR334.

On the cooperativity of CHIP with other E3s, two action
modes have been proposed: 1) in neurons, CHIP acts with Fbx2
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The latter case bears some similarity with the current study in
that the substrate (GHR) binds two E3s, independently. How-
ever, a major difference is that in both models CHIP is involved
in ubiquitin/proteasome dependent degradation, whereas in
the current study CHIP and SCFβTrCP function in cargo selec-
tion for endocytosis. Indeed, we cannot exclude the possibility
that in the course of this process, an as yet unknown (Lys48-
ubiquitinated) substrate must be degraded to allow progression
of the process, as we hypothesized previously (55). CHIP-
Ubc13-Uev1a may work sequentially with SCFβTrCP, whereby
the SCF ligase initiates ubiquitination, and the CHIP enzyme
complex carries out Lys63-linked polyubiquitination on an as
yet unidentified substrate (56). It was recently shown that
CHIP/Ubc13 can extend a monoubiquitinated protein and syn-
thesize Lys63-linked chains on these ubiquitin moieties (17). As
in the absence of Ubc13/CHIP, where GHR is ubiquitinated to
the same extent as in clathrin-depleted cells, it might indicate
that Ubc13/CHIP might edit pre-existing, βTrCP-derived,
ubiquitin chains on GHR. The opposite could be envisioned if
we consider that the WD40 propeller domain of βTrCP was
identified as a ubiquitin-binding domain, indicating that
βTrCP might also recognize a ubiquitin moiety as its substrate
(57). However, if we deplete CHIP, GHR is still ubiquitinated,
presumably by βTrCP. Thus, CHIP activity is not required for
GHR ubiquitination by βTrCP, suggesting that CHIP does not
initiate this ubiquitination. Together, these observations lead
to a model in which SCFβTrCP binding to the GHR initiates
the endocytosis followed by the CHIP activity that results in clath-
рин-mediated endocytosis.

CHIP is known to function in the molecular chaperone sys-
tem together with Hsp90 and Hsp70 (58, 59). For the mineralo-
corticoid receptor, it was reported that inhibition of Hsp90
results in increased CHIP-mediated ubiquitination and subse-
quent proteasomal degradation of the receptor (60). In another
study, by Wang et al. (20), it was shown that Hsp70 competes
with SMAD1/5 for the TPR-mediated binding of CHIP and
subsequent ubiquitination of SMAD1/5. These examples show
that under certain stress conditions affecting the activity of
Hsp70/90, the activity of CHIP toward other substrates might
increase. In such a model, higher cellular concentration of
“free” CHIP might accelerate GHR endocytosis and negatively
regulate the GH sensitivity of cells.
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