The Ubiquitin Ligase SCF(βTrCP) Regulates the Degradation of the Growth Hormone Receptor*

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SCF ubiquitin ligases play a pivotal role in the regulation of cell division and various signal transduction pathways, which in turn are involved in cell growth, survival, and transformation. SCF(TrCP) recognizes the double phosphorylated DSGDβXS destruction motif in β-catenin and IκB. We show that the same ligase drives endocytosis and degradation of the growth hormone receptor (GHR) in a ligand-independent fashion. The F-box protein β-TrCP binds directly and specifically with its WD40 domain to a novel recognition motif, previously designated as the ubiquitin-dependent endocytosis motif. Receptor degradation requires an active neddylation system, implicating ubiquitin ligase activity. GHR-βTrCP binding, but not GHR ubiquitination, is necessary for endocytosis. TrCP2 silencing is more effective on GHR degradation and endocytosis than TrCP1, although overexpression of either isoform restores TrCP function in silenced cells. Together, these findings provide direct evidence for a key role of the SCF(TrCP) in the endocytosis and degradation of an important factor in growth, immunity, and life span regulation.

Understanding how metabolism of cells and organisms is regulated and how this connects to the regulation of longevity, cell cycle, apoptosis, and immunity are major challenges of modern biology (1). Growth hormone (GH)2 and its receptor (GHR) act at these crossroads as becomes apparent in the current study in which we show that a regulator of cell cycle and immunity appears to regulate GH sensitivity of cells. All cells of the human body contain GHRs. Upon GH binding, these receptors initiate signal transduction that results in expression of genes involved in anabolic processes, including increased protein synthesis, lipid degradation, immune function, muscle mass, bone turnover, and tooth development (2). The GHR was the first class one cytokine receptor to be cloned (3). Signal transduction of class-one cytokine receptors recruits members of the Janus kinase family tyrosine kinases that bind to a proline-rich motif in the intracellular membrane-proximal domain, the box 1 motif (4). For the GHR, Jak2 acts as initiator of several signaling cascades, including Stat activation. Box 1-related activities contribute little to GHR endocytosis (5).

For receptor tyrosine kinases, both signal transduction and receptor abundance are regulated by their ligands: epidermal growth factor, c-kit, insulin and Notch binding initiate not only signal transduction, they also induce a swift degradation of their receptors. For cytokine receptors, these two parameters seem to have their own independent regulating mechanism. There is good evidence that the GH sensitivity of cells is mainly regulated via the ubiquitin-dependent endocytosis motif (UbE motif) in the cytokine receptor box 2 region (6). Mutation of the UbE motif (DDSWVEFIELD) leads to a dramatic drop in endocytosis and lysosomal degradation, rendering the cells more GH-sensitive.

The ubiquitin-proteasome system provides the major pathway for nonlysosomal degradation (reviewed in Ref. 7). The F-box protein β-transducing repeat-containing protein (βTrCP) serves as the substrate recognition subunit in the SCF(βTrCP) ligase (8). Classical examples of substrates are β-catenin, NFκB, and IκB (inhibitor of NFκB). Two isoforms exist; βTrCP1 is mainly present in the nucleus, whereas βTrCP2 resides in the cytosol (9). These ligases play a pivotal role in cell division and various essential signal transduction pathways for tumorigenesis. Their substrate binding motifs consist of seven propeller-shaped WD40 domains that specifically bind short motifs in its central cavity. These motifs generally are 6 amino acid residues long with a common structure of DSGXX2+,S. To be recognized by the SCF(βTrCP), both serine residues need to be phosphorylated. Recently, it became evident that, for a restricted number of plasma membrane proteins, ubiquitination triggers internalization and vacuolar/lysosomal rather than proteasomal degradation (reviewed in Ref. 10). This pathway is best understood in yeast, where a number of plasma membrane proteins are endocytosed in an ubiquitin-dependent manner (11, 12). Several studies have shown that monoubiquitination of plasma membrane proteins is sufficient to stimulate their endocytosis (11, 13). In mammalian cells, the GHR, the β-adrenergic receptor, and the epithelial sodium channel, ENaC, are examples of membrane proteins that endocytosis in an ubiquitin system-dependent manner (14–16). In some cytokine receptors, the conserved DSGX2+,S motif serves as a degradation signal via the SCF(βTrCP) ligases. Both for the interferon-γ and the PrL receptor, it was shown that this

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‡The abbreviations used are: GH, growth hormone; GHR, growth hormone receptor; UbE, ubiquitin-dependent endocytosis; GST, glutathione S-transferase; mAb, monoclonal antibody; siRNA, small interfering RNA; GFP, green fluorescent protein; E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin carrier protein; ER, endoplasmic reticulum; βTrCP, β-transducing repeat-containing protein.

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E3 is involved in their degradation. An important finding in these studies was that receptor degradation mainly depends on ligand binding; in addition, phosphorylation at both serine residues of the degron was required (17–19).

In the present study, we show that the UbE motif, deviant from the canonical βTrCP recognition motif, is specifically recognized by a functional SCF(βTrCP) complex and that this interaction is instrumental in the endocytosis and degradation of the GHR. Ubiquitination of the GHR is not required, since βTrCP silencing slows degradation of the GHR without lysine residues in the cytosolic tail as efficiently as wild type GHR.

EXPERIMENTAL PROCEDURES

Materials—βTrCP1 and βTrCP2 cDNA in pcDNA3 expressing the FLAG-tagged proteins were generous gifts of Tomoki Chiba (Tokyo Metropolitan Institute of Medical Science). Full-length rabbit GHR cDNA in pcDNA3 was described (20). GST fusion proteins expressing GST-βTrCP (271–334), and GST-βTrCP (271–318) were produced as described (21). pcDNA-GHR constructs used for alanine scanning of the UbE motif and GHR (1–399), K271–362R, designated as GHR399-Kallir, were constructed as described before (22). The following siRNA sequence sets were used to silence the following: both βTrCP1 and -2, GUGGAUUAUGUGGAAACU(Ct) (called “combi probe”); βTrCP1 only, GAUAUACCAGAGAGAAGA-Utt; βTrCP2 only, GAGGCCAUCAGAAGGAAACtt; clathrin heavy chain, CCAUGGCGCGUGGAAGAUU; GFP and HPV E6/E7 (used as controls), GGCUCAGGCGAGGCGCACC and CUAAUCACACUGGGUUAUtt, respectively. Dharmaco siGENOME SMARTpool’s BTRC NM-003939 was used to silence βTrCP1, and FBXW11 (NM-012300) was used to silence βTrCP2. QuikChange mutagenesis primers to introduce three silent mutations in the target sequence of βTrCP1 for combi TrCP, 5′-TGGTCAGAGTCAGATCAAGTGGAG-TGGTCTGAATCAGATCAAG-3′, and to introduce three silent mutations in the target sequence of TrCP2 for combi βTrCP, 5′-TGGTCTGAATCAGATCAAG-TGGAGTTCCGTCGAACATTTATCCCCAATGTG-3′. Chemically synthesized siRNA duplexes were purchased from Ambion (Austin, TX). Antibody, recognizing the cytoplasmic domain of the GHR, was raised against amino acids 271–318 of the cytosolic tail of the GHR (23). Antibody (mAb 5) against the extracellular domain of the GHR was from Agen (Acadia Ridge, Australia), goat anti-mouse IgG Alexa680 was from Molecular Probes, Inc., and goat anti-rabbit IgG IRDye800 was from Rockland Immunochemicals Inc. (Gilbertsville, PA). Mouse monoclonal anti-FLAG (M2) was from Sigma. The polyclonal anti-βTrCP1 serum was raised in rabbits against a βTrCP1 peptide consisting of amino acids 1–93 fused to glutathione S-transferase. The fusion protein was produced by cloning the DNA encoding amino acids 1–93 of TrCP1 into vector pGEX1At, after which the construct was expressed in Escherichia coli. Immobilized streptavidin was from Pierce, glutathione-Sepharose was from Amersham Biosciences, and Ni²⁺-nitrilotriacetic acid-agarose was from Qiagen. Culture media, fetal calf serum, l-glutamine, and antibiotics for tissue culture were purchased from Invitrogen.

Cell Culture—Hek293 and ts41 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Hek293 cells stably expressing the wild type GHR (Hek-wtGHR) were grown in the same medium supplemented with 0.6 mg/ml Geneticin (G418; Invitrogen). All cells used were grown at 37 °C with 5.0% CO₂. Twice a week, cells were washed with phosphate-buffered saline (PBS), detached from the flask with trypsin-EDTA (Invitrogen), diluted in fresh growth medium, and split into new culture flasks.

Transfections—Cells were transfected using Lipofectamine 2000 (Invitrogen). Seventy percent confluent cultures were transfected with 0.2–0.9 μg of DNA in 12-well plates according to the manufacturer’s protocol. Transfected cells were used for Western blot experiments and 125I-GH binding experiments 1–2 days after transfection. For fluorescence microscopy cells were transfected with Eugene-6 (Roche Applied Science).

To silence the expression of both βTrCPs, Hek-wtGHR cells were transfected with small interfering RNA (siRNA) using Lipofectamine 2000 according to the description of the manufacturer; 3 days after transfection, cells were used for Western blot and 125I-GH binding experiments. Control cells were transfected with siRNA-GFP. To control the effect of the gene expression silencing, Hek293 cells were transiently transfected with a pcDNA3-FLAG-βTrCP. Western blots were analyzed using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

125I-GH Binding and Internalization—125I-Human GH was prepared using chloramine T (20). For internalization experiments, cells, grown in poly-D-lysine-coated 12-well plates, were washed with 1 ml of minimal essential medium supplemented with 20 mM Hepes, pH 7.4, and 0.1% bovine serum albumin, and incubated at 37 °C in a water bath for 1 h. After washing the cells three times with ice-cold PBS-complete (PBS-c: PBS with 0.135 g/liter CaCl₂ and 0.1 g/liter MgCl₂), 0.75 ml 125I-GH (180 ng/ml in Mem/Hepes/0.1% bovine serum albumin) was added to the cells. 125I-GH was bound for 2 h on ice. Unbound 125I-GH was aspirated and the cells were washed three times with PBS-c. Cells were incubated 10 min at 37 °C in minimal essential medium, Hepes, 0.1% bovine serum albumin to allow receptor internalization and washed three times with ice-cold PBS-c. Membrane associated 125I-GH was removed by treating the cells twice with 750 μl acid wash (0.15M NaCl, 50 mM glycine, 0.1% bovine serum albumin, pH 2.5) for 5 min on ice. Acid wash was collected in counting tubes, and radioactivity was measured using a LKB γ counter. Cells were solubilized overnight in 1 N NaOH. Internalized 125I-GH was determined by measuring the radioactivity in the collected NaOH. Unspecific counts were determined by incubating the cells with 125I-GH together with excess unlabeled GH (9 μg/ml). Internalization is expressed as a percentage of the total specific radioactivity after 10 min at 37 °C.

In Vitro Binding Assay—Wild-type and mutant GHR proteins were expressed in Hek293, and the cells were lysed in 50 mM Tris-HCl (pH 7.5), 0.15 or 1.0 mM NaCl, 50 mM NaF, and 0.5% Nonidet P-40. The receptors were purified with biotinylated GH and streptavidin beads and stringently washed with lysis
buffer. The beads were incubated with in vitro translated and 35S-labeled βTrCP2 for 60 min at 4 °C and extensively washed with lysis buffer, and associated proteins were analyzed by SDS-PAGE and autoradiography. cDNA of βTrCP2 constructs in pcDNA3.1/FLAG was used in an in vitro transcription translation system supplemented with [35S]methionine and TNT®-T7 coupled reticulocyte lysate ready reaction mix according to the instructions of the manufacturer (Promega). Binding to GHR-(1–286) was taken as background. To analyze GHR-containing protein complexes at the cell surface, the cells were incubated with biotinylated GH on ice. To recover all GHR-protein complexes from cells, the cells were first lysed and then incubated with biotinylated GH. Equal amounts of cell lysates were incubated for 1 h at 4 °C with equal amounts of GST-GHR-(271–334) or GST-GHR-(271–286) bound to GSH beads. Beads were pelleted in an Eppendorf centrifuge and washed three times with 1 ml of lysis buffer and twice with PBS containing protease inhibitors. The beads were boiled in 2× sample buffer. Samples were subjected to SDS-PAGE. Proteins associated with the GST fusion proteins were visualized by autoradiography. To analyze equal loading of the fusion proteins, the gels were stained with Coomassie Brilliant Blue and analyzed using an Odyssey imaging system.

Production of GST-GHR Fusion Proteins—pGEX-GHR plasmids were used to express GST-GHR fusion proteins in E. coli (strain BL21) (21). The synthesis of recombinant proteins was induced by isopropyl-1-thio-β-d-galactopyranoside. GST fusion proteins were purified with GSH-beads (Amersham Biosciences) by the procedure recommended by the manufacturer.

Immune Fluorescence Microscopy—Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier’s instructions (Amersham Biosciences). Transfected cells, grown on coverslips, were incubated for 15 min with Cy3-GH (1 μg/ml) and/or Alexa488-labeled transferrin (5 μg/ml). Cells were washed with PBS to remove unbound label and fixed for 2 h in 3% paraformaldehyde in phosphate-buffered saline. After fixation, the cells were embedded in Mowiol, and con-
focal laser-scanning microscopy was performed using a Leica TCS 4D system.

RESULTS

Expression of βTrCP Is Required for GHR Endocytosis—Endocytosis of the GHR depends on the ubiquitination machinery, implicating the involvement of an ubiquitin ligase. Since recent studies show that βTrCP is involved in the degradation of certain cytokine receptors, we used small interfering RNA to silence both βTrCP isoforms and study the effects on GHR turnover. Fig. 1A shows that siRNA for βTrCP inhibits GH uptake to the same extent as silencing of clathrin heavy chain: ~50% as compared with nonrelevent siRNA(GFP). In this experiment, the clathrin heavy chain Western blot signal was reduced to 4% compared with siRNA(GFP)-treated cells. Expression of co-transfected FLAG-tagged βTrCP2 was reduced to 10% using the “combi” silencing oligonucleotides that silence both TrCP1 and TrCP2 (Fig. 1B). We conclude that βTrCP is involved in endocytosis of the GHR.

At steady state, almost all (mature, 130-kDa) GHRs are present at the cell surface. To visualize the effect of βTrCP silencing on endocytosis, we incubated the cells with both Cy3-labeled GH and Alexa-488-labeled transferrin. Fig. 1C shows that silencing both βTrCP isoforms resulted in a strong increase of Cy3-GH at the plasma membrane, whereas transferrin recycling remained intact, excluding a general effect on clathrin-mediated endocytosis. These results demonstrate that βTrCP is an obligatory part of the GHR endocytosis machinery. Since both GH and transferrin are endocytosed via clathrin-coated vesicles (24), it suggests that βTrCP is part of the cargo selection complex for the GHR.

An obvious consequence of endocytosis inhibition is an accumulation of GHRs at the cell surface. In Fig. 1D, lanes 1 and 2, we used Hek293 cells transfected with GHR DNA and silenced for both βTrCP isoforms. To measure the amount of GHRs at the cell surface, the cells were incubated with biotin-GH, and the GH-GHR complexes were isolated and quantitated. siRNA(βTrCP) treatment caused a 2–3-fold increase in GHR at the cell surface. To ascertain that the increase was not due to a silencing effect on the amount GHRs synthesized, metabolic labeling with [35S]methionine was performed and showed that the GHR synthesis was not changed (not shown). The cellular concentration of βTrCP1 was measured on Western blots (Fig. 1D). By this method, using an optimized protocol for βTrCP, we obtained ~90% silencing. Using this silencing oligonucleotide, βTrCP2 was silenced to the same extent (Fig. 1B). Previous studies have shown that by inhibiting GHR endocytosis through (mutation of) the Ube motif, maximally 60% inhibition of GHR endocytosis was achieved compared with removal of the GHR tail, including the complete Ube motif. Apparently, both βTrCP and clathrin silencing were insufficient to overcome this half-maximal effect on GHR turnover. Combining the two siRNAs did not result in more endocytosis inhibition. In agreement with our previous experience, these effects are too limited to measure half-life differences in dynamic experiments successfully (e.g. using pulse-chase protocols) (21).

As an important control, based on our previous observations that the GHR itself is not an obligatory ubiquitination target, we used a GHR truncated at amino acid residue 399, in which all 10 lysines are replaced by arginine residues (GHR(1–399) K271–362R) (22). Fig. 1D, lanes 3–6, shows that TrCP silencing induces a 2–3-fold increase in cell surface GHRs whether or not lysine residues are present in the receptor tail. To control the experiments of Fig. 1D for equal transfection efficiencies, we co-transfected GFP DNA in all situations and analyzed the cell lysates for GFP; under all (silencing) conditions and various DNA constructs, the transfection efficiency was identical within measurement limits (Fig. 1D, bottom).

Since we previously concluded that GHR internalization requires the recruitment of the ubiquitin conjugation system to the GHR Ube motif rather than the conjugation of ubiquitin to the GHR itself, we now conclude that βTrCP is a prime candidate to act as E3.

Other Factors of the βTrCP–GHR Complex—Once we established that βTrCP is involved in GHR endocytosis and degradation, we asked whether βTrCP is in a protein complex with the GHR. Pull-down experiments with biotin-GH, depicted in Fig. 2A, show that both co-expressed full-length (lane 4) and mutant TrCP without the F-box domain (lane 5) were present in the isolated complexes. If His6-TrCP1 was used to isolate the protein complex (Fig. 2B), both ER and cell surface GHR were pulled down (lane 4). The interaction occurs intracellularly, as shown in an experiment in which GHR and His6-TrCP1 were expressed in different plates (lanes I and 5) and mixed after cell lysis (lane 3). Under these conditions (low cellular concentrations, low temperature, and short time periods) βTrCP does not interact with GHR. Thus, TrCP is in a protein complex with GHR, probably already in the ER.

βTrCP generally acts as subunit of a SCF ubiquitin ligase. To examine the functional complex in GHR endocytosis, we co-transfected βTrCP2 and Skp1 together with the GHR. Pull-down experiments with biotinylated GH and Western blot analysis of the isolated complex revealed that Skp1 interacted with GHR only in the presence of βTrCP (Fig. 2A, lane 6). In the absence of βTrCP (lane 6) or in the presence of βTrCP lacking the F-box (lane 5), no Skp1 was isolated. In lanes 10 and 11, we repeated the experiment and asked whether Skp1 might have associated with the GHR protein complex after lysis. As for βTrCP, the interaction does not occur if cell lysates from cells, expressing the components separately, were mixed (M). Together, this implies that GH, GHR, TrCP2, and Skp1 are in the same protein complex.

To analyze the involvement of an active SCF complex, we took a genetic approach and expressed GHR in hamster ts41 cells. These cells contain a temperature-sensitive mutation in the NEDD8 activation enzyme, APP-BP1, and fail to degrade substrates via E3s with Cullin family members (e.g. p27 via the SCF(skp2) (25), due to defective neddylation. Thus, the E3-SCF(TrCP) is itself regulated via covalent modification of the ubiquitin-like protein Nedd8 by UBC12, whereas deneddylation is performed by the nonsosmolesome complex. This modification stabilizes the E2-Cullin/Rbx1 interaction and in turn stimulates ubiquitination of the target substrates. Fig. 2C shows that Cy3-GH is endocytosed at the permissive temperature but
largely remains at the cell surface at the nonpermissive temperature (39°C). In the same experiment, Alexa488-labeled transferrin entered the cells undisturbed, although there seemed to be a different distribution intracellularly. This shows that an intact neddylation system is specifically required for uptake of the GHR. Together with the results on βTrCP and Skp1, these data strongly suggest that GHR endocytosis requires a fully functional SCF(βTrCP) complex.

βTrCP Interacts with the GHR Tail via the UbE Motif—To further examine the role of βTrCP in GHR endocytosis, we analyzed βTrCP2, since it is dominantly active in the cytosol (26). Hek293 cells were co-transfected with FLAG-tagged βTrCP2 together with GHR or the truncation GHR-(1–286) (Fig. 3A). GHR-(1–286) still contains box 1, the attachment site for Jak2, but lacks the UbE motif. This receptor is unable to endocytose and, therefore, accumulates at the cell surface, resulting in a high amount of mature receptor (55 kDa) compared with its 40-kDa ER form (Fig. 3B, left, lane 2). The wild-type GHR migrates at 130 kDa, whereas its precursor ER form resulting in a high amount of mature receptor (55 kDa) compared with its 40-kDa ER form (Fig. 3B, left, lane 2). The wild-type GHR migrates at 130 kDa, whereas its precursor ER form appears as a doublet at 110 kDa (lane 1). Cell lysates were incubated with biotinylated GH, and the protein complexes were isolated with biotin-GH/streptavidin beads (lanes 3 and 4) and analyzed on blot using anti-FLAG. Lanes 1 and 2, loading control GHR (mAb 5); lanes 5 and 6, loading control βTrCP (arrow). (B) Figure 3B, right). 10 cm plates of Hek293 cells were transfected with equal amounts of radioactive βTrCP. Data are representative of three experiments. WB, Western blot.

for Jak2, but lacks the UbE motif. This receptor is unable to endocytose and, therefore, accumulates at the cell surface, resulting in a high amount of mature receptor (55 kDa) compared with its 40-kDa ER form (Fig. 3B, left, lane 2). The wild-type GHR migrates at 130 kDa, whereas its precursor ER form appears as a doublet at 110 kDa (lane 1). Cell lysates were incubated with biotinylated GH, and the protein complexes were isolated with streptavidin beads. Comparing the full-length GHR with GHR truncated at 286 shows that co-transfected FLAG-labeled βTrCP2 was only isolated if full-length GHR was present (Fig. 3B, left, lanes 3 and 4). In Fig. 3B, right, we used various GHR truncations and mutations to pinpoint the tail segment involved in βTrCP binding. The receptors were purified from transiently transfected Hek293 cells, via biotinylated GH and streptavidin beads, and then incubated on ice with equal amounts of in vitro synthesized 35S-labeled βTrCP. Wild-type GHR and GHR truncated at 434, 369, or 334 bound ~16% of the input 35S-labeled TrCP. GHRs truncated at 330, 326, and 286 as well as the UbE mutants GHR-D331A and GHR-F327A showed little binding. Mutation of the serine residues in the
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DSGRTS motif of the full-length GHR (GHR-S366,370A) did not affect the GHR-SCF(TrCP) interaction (Fig. 3B, right, lane 8).

**Similar UbE Motif Specificity for GHR-βTrCP Interaction and GHR Endocytosis**—Previously, we have shown that endocytosis of the GHR depends both on a functional ubiquitination system and on an intact UbE motif (20, 27, 28). Using mutational analysis of the UbE motif by changing every single amino acid residue into alanine, the contribution of each amino acid was determined. The experiment identified the key residues in the UbE motif (DDSWVEFIELD) for endocytosis. As shown in Fig. 1, βTrCP is an obvious factor to act as a regulator of GHR endocytosis. Therefore, we used the pull-down assay of Fig. 3B (right) to ask whether βTrCP binds to the GHR in a specific fashion. Each of the amino acids 320–334 were changed in alanine residues, and the 15 GHR constructs were tested in Hek293 cells. The cells were transfected with equal amounts of the different constructs and showed comparable GHR expression judged by the amounts of high mannose (ER-localized) GHR (not shown). Western blot analysis of the cell extracts shows that the mutations E326A, F327A, I328A, and D331A caused a significant increase in mature (130-kDa) GHR (Fig. 4A, indicative of impaired degradation. Biotin-GH was used to isolate the GH-GHR complexes from the cell surface, and the GHRs were incubated with equal amounts of 35S-labeled βTrCP. Most mutations resulted in inhibition of βTrCP binding as compared with wild-type GHR. However, mutation of the same 4 amino acids inhibited the βTrCP binding more than 60% (Fig. 4B). Compared with the mutational analysis previously performed for endocytosis (see Fig. 4C and Fig. 4 in Ref. 22), we note a striking similarity in patterns; the same 4 amino acid are important for endocytosis of the GHR (27).

**GHR Binds to the WD-40 Domain of βTrCP**—βTrCP canonically binds to the DSGFΔXS motif in IκB, β-catenin, and human immunodeficiency virus vpu after both serine residues are phosphorylated. To characterize the βTrCP-GHR binding further, we used bacterially produced GST fusion proteins containing only the 65 membrane-proximal amino acid tail residues and in vitro synthesized 35S-βTrCP (Fig. 5A) (21). Fig. 5B (left, lanes 2) shows that both βTrCP1 and βTrCP2 bind with high efficiency to the GST-GHR-(270–334). If a slightly shorter fusion protein was used (residues 270–318), the interaction was virtually abolished (lanes 3). This demonstrates an efficient interaction between βTrCP and the UbE motif in the GHR. Since the binding is highly efficient at 0 °C, the interaction is very likely direct and does not require any other post-translational modification. The next question is whether GHR binds to βTrCP via its WD40 domain, as has been established for other E3 substrates. Four different constructs were made, expressing βTrCP2 segments 1–174, 1–228, 174–542, and 228–542 (Fig. 5A). Using the GST-GHR fusion proteins, in vitro binding assays clearly show that the interaction is via the WD40 domain; the F-box is not necessary for interaction (right). In addition, specific binding was only observed if the UbE motif was present (compare lanes 2 and 3).

Together, the experiments show that βTrCP interacts with the GHR and that the F-box protein is required for GHR endocytosis. From comparison between the mutational analyses previously measured for GHR endocytosis and the results obtained with the same constructs for GHR-βTrCP interaction, we conclude that βTrCP is a prime regulator of GHR endocytosis via the UbE motif.

**βTrCP Isoforms**—Mainly due to lack of specific antibodies, until now the role of βTrCP has not been elucidated at the level of isoform specificity. The main differences between the isoforms are located at the N terminus. βTrCP1, in general acting on substrates involved in cell cycle regulation, is mainly present in the nucleus, whereas βTrCP2, presumably acting on membrane-bound substrates, resides in the cytosol (9, 29). Our experiments show that both isoforms can be detected in pull-down experiments from bacterially produced GST-GHR fusion proteins (Fig. 5B). However, only functional experiments can validate the interaction. Therefore, we determined the effect of
isoform-specific βTrCP silencing on GH internalization. We used several (combinations of) silencing oligonucleotides to specifically deplete transiently expressed FLAG-tagged βTrCP isoforms. Fig. 6 A (bottom) shows that the siRNA(TrCP) (combi) probe silenced both isoforms, whereas siRNA(TrCP-1) only silenced TrCP1 and not TrCP2. siRNA(TrCP2) silenced only TrCP2 and not TrCP1, whereas the combination of both TrCPs (TrCP1 + 2) silenced both isoforms. From the upper panel of Fig. 6 A, we conclude that silencing of βTrCP1 alone does not affect GH internalization, whereas in every combination in which βTrCP2 was silenced, a clear effect on GH uptake was visible, comparable with silencing of the clathrin heavy chain.

In Fig. 6 B, we asked whether replenishment of either of the isoforms is capable of restoring the silencing phenotype. The lower panel shows that silencing is specific; the combi siRNA probe silences both isoforms (lanes 3), except if a βTrCP siRNA-resistant replacement vector was used (lanes 4). If βTrCP1- or βTrCP2-specific silencing probes were used, as expected, the “resistant” βTrCP DNA could be silenced (lanes 5–8). Using this strategy, the GH internalization assay shows that overexpression of both resistant TrCP isoforms can rescue the silencing effect, indicating that both βTrCP isoforms are capable of GHR cargo selection at the cell surface. Our conclusion is that, although both βTrCP isoforms are capable of acting in GHR endocytosis, βTrCP2 is the most obvious candidate for this role in endogenous situations.

DISCUSSION

Previously, we have shown that GHR endocytosis and subsequent degradation in the lysosomes depends on the activity of the ubiquitin system via the UbE motif. Here, we identify βTrCP as a key factor required for GHR endocytosis. βTrCP specifically binds to the GHR motif, which is required for ubiquitination-dependent endocytosis. These data support the conclusion that this factor recruits the ubiquitination machinery. The results with Skp1 and neddylation strengthen our hypothesis that the complete SCF(βTrCP) complex, including Skp1, Cul1, Rbx1, and an ubiquitin conjugase, is present at the GHR tail. This is the first observation that shows direct involvement of a SCF E3 in endocytosis of cytokine receptors without targeting the receptor itself for ubiquitination. Although the test system uses GHR-(over)expressing cells, the experiments show that endogenous TrCP levels are sufficient to rapidly initiate GHR endocytosis and degradation. The
physiological relevance of the findings is further supported by the TrCP silencing experiments; if the cellular levels of both TrCP species were diminished, uptake and degradation of GHR were inhibited.

Detailed structural information is available on the interaction of the WD40 domain of SCF(βTrCP) and β-catenin (30). The F box protein βTrCP recognizes the dual phosphorylated DpSGFXpS destruction motif (where pS represents phosphoserine), present in β-catenin, and directs the SCF(βTrCP) E3 to ubiquitinate it at a lysine residue, 13 amino acids upstream of the destruction motif (commonly present 9–14 positions upstream). The β-catenin peptide binds the top face of the β-propeller, with the 6-residue destruction motif dipping into the central channel. All seven WD40 repeats contact β-catenin. Comparison with the UbE motif reveals little resemblance to the catenin destruction motif: the UbE motif is 11 amino acids long, it contains 3 acidic residues critical for binding (Asp321, Glu326, and Asp331) instead of the two phosphorylated serine residues in β-catenin, and it lacks the canonical glycine residue, present in all phosphorylated DSGFXS destruction motifs described until now (31, 32). The results of Fig. 4 are most predictive and point to a binding site with EFLXAD as the strongest interacting residues. Another deviation from the canonical motif is the lack of a lysine residue at positions 9–14 upstream, although there is a conserved lysine at 315 immediately after a tyrosine that is phosphorylated upon GH binding. However, mutation of this lysine residue into arginine does not affect its ubiquitination-dependent endocytosis (22). Together, these results are in line with our previous observation that ubiquitination of the GHR is not required for endocytosis. Thus, binding of a presumed SCF(βTrCP) E3 does not target the GHR for ubiquitination but must be directed toward another factor in the GHR-ubiquitination complex (22). Even without a three-dimensional structure of the precise interaction between the βTrCP-WD40 domain and the UbE motif, it is already clear that the UbE-WD40 interaction considerably differs from the WD40-DSGFXS destruction motif interactions described until now.

Interaction of βTrCP is the first step toward factual endocytosis of the GHR via a clathrin-mediated selection process. To involve the ubiquitination machinery in this process, the GHR must be dimerized (33). Recent observations on βTrCP show that it indeed contains a dimerization domain that is important for its E3 function (34). Also, cullins seem to occur as dimers in the context of SCF complexes (35). Our in vitro binding experiments with GST-GHR tails suggest that also monomeric GHR can bind βTrCP, although due to the tendency of GST to homodimerize, it may well be that also the GST-GHR molecules represent dimeric GHR segments (36). Previously, we reported that a chimeric GHR consisting of the extracellular domain of low density lipoprotein receptor-like protein and the trans-membrane and cytosolic domain of the GHR could not dimerize anymore and that monomeric GHR can endocytose in an ubiquitin system-independent fashion. Since dimerization occurs in the ER and βTrCP is a homodimer, it is possible that βTrCP acts as a chaperone at the ER. Therefore, it remains to be investigated whether the dimerization of βTrCP poses the necessity for dimerization on the GHR.

Cargo selection via βTrCP thus requires an active E3, followed by proteasomal activity, and interaction with the clathrin-mediated machinery (22). Whether the SCF E3 ubiquitinates a factor X and enables GHR to connect to the clathrin heavy chain remains to be determined. Previously, we have identified several factors that bind specifically to the UbE motif (21). The tetrapeptide repeat-containing protein SGT may be a factor bridging the gap between βTrCP and clathrin via the U-box ligase carboxyl terminus of Hsc70-interacting protein (21, 37). Another scenario may be that the ubiquitin moiety present in the SCF(βTrCP) complex serves as a ligand for ubiquitin-binding proteins that are part of the clathrin-mediated endocytosis machinery (38).

βTrCP is involved in the degradation of prolactin and interferon receptors (17–19). Fuchs et al. (31) identified βTrCP interaction with these receptors via their DSGFXS destruction motifs and showed that the E3 ubiquitinates both the interferon-γ and prolactin receptors, dependent on an intact DSGFXS motif. Upon ligand binding, this motif is phosphorylated, after which the SCF E3 ubiquitinates certain lysines in the tail of the receptors. This leads to their destruction. It is clear that this scenario differs from the action of βTrCP in GHR endocytosis. Although the GHR does contain a DSGFXS-like destruction motif (DSGRTS), mutation of the serine residues did not result in a shorter half-life or in inhibition of GHR endocytosis; nor did mutation/removal of the motif interfere with the βTrCP-GHR interaction.

Ubiquitination by the SCF(βTrCP) E3 of a factor generally results in its destruction by the proteasome. In most cases, the decision to degrade the target is not in the SCF(βTrCP) E3 action but depends on the activity of one or two kinases that phosphorylate serine residues in the degradation motif. Our experiments with GST-GHR fusion proteins indicate that binding of βTrCP to the UbE motif does not depend on a preceding action of another post-translation modification. Thus, very likely, the cytosolic concentration of βTrCP determines its activity level to induce GHR endocytosis. This is reflected in several experiments showing that decreased amounts of βTrCP inhibit GH uptake and GHR degradation. Thus, the free cytosolic concentration of βTrCP may function as a factor in GH sensitivity of cells; at low concentrations, the cells are more sensitive to GH than at high concentrations. Currently, βTrCP appears to be involved in the degradation of at least 10 substrates at the same time: β-catenin, NFκB, IκB (31), Emi1 (39), Wee1 (40), ATF4 (41), HDl (42), CDC25B (32), IFN-R1 (17), Gli3 (43), and Prl-R (21). In all except for CDC25B, degradation is regulated by phosphorylation. Although the two βTrCP sub-species may be differently localized in the cell, they can take over their tasks if one of the two is silenced by RNA interference techniques. Since probably no modification is required for βTrCP to bind GHR, the two proteins may regulate each other’s activity; more cellular βTrCP results in increased GHR endocytosis/degradation, whereas more GHR may absorb free βTrCP, immediately affecting the other βTrCP functions. Evidence for this comes also from the observation that heteroge-

3 P. van Kerkhof, J. Putters, and G. J. Strous, unpublished results.
neous nuclear ribonucleoprotein-U acts as a pseudosubstrate (44). Generally, βTrCP is part of an E3 complex involved in degradation of key factors controlling cell growth, survival, and transformation (31). Since it is a short lived protein itself, more studies are needed to understand the consequences of βTrCP concentrations for cellular regulation and the GHR activity in particular.

The GHR-IGF1 system is involved in longevity, cell cycle, apoptosis, and immunity (1); in catabolic (cachectic) conditions, such as old age and cancer, unknown stress signals turn the system off, resulting in GH insensitivity, whereas anabolic conditions require cells to be highly GH-responsive. Whether and how βTrCP is at this balance remains to be elucidated.

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