Small glutamine-rich tetratricopeptide repeat-containing protein (SGT) interacts with the ubiquitin-dependent endocytosis (UbE) motif of the growth hormone receptor

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Endocytosis of the growth hormone receptor (GHR) is regulated by the ubiquitin-conjugating system. A cytosolic 10 amino acid motif, referred to as the ubiquitin-dependent endocytosis (UbE) motif, is involved in the ubiquitination as well as in the endocytosis of the receptor. Proteins that are implicated in one of these processes have not been identified so far. Using a glutathione S-transferase (GST)-pull-down assay with a GST fusion protein encompassing the UbE motif of the GHR, a 35 kDa protein was purified. The protein was identified by MS as small glutamine-rich tetratricopeptide repeat (TPR)-containing protein (SGT). We found that GHR interacts with SGT. *In vivo*, both the precursor and the mature form of the receptor interacted with

SGT. Inactivation of the ubiquitin-conjugating system did not affect the GHR–SGT interaction. Binding studies showed that the first TPR motif of SGT interacts with the UbE motif of the GHR. Taken together, these data show that SGT is a GHR-interacting protein, which binds independent of the ubiquitin-conjugating system.

Key words: chaperone protein, growth hormone receptor, mass spectrometry, protein–protein interaction, small glutamine-rich tetratricopeptide repeat-containing protein (SGT), tetratricopeptide repeat.

INTRODUCTION

Growth hormone (GH) regulates a wide range of processes, including somatic growth, metabolism and cellular differentiation, that are mediated by the GH receptor (GHR) [1]. GHR was the first of the class I cytokine receptors to be cloned [2]. Members of this family, including receptors for erythropoietin, prolactin, thrombopoietin and several interleukins, share a number of structural characteristics [1,3,4]. The extracellular domain contains four paired cysteine residues and a Trp-Ser-Xaa-Trp-Ser motif (where Xaa represents any amino acid) involved in ligand binding [5]. In the intracellular domain, sequence homology is restricted to two motifs box 1 and box 2. Both motifs are localized to the membrane-proximal domain of the cytosolic tail and are known to be involved in signal transduction. Cytokine receptors lack intrinsic kinase activity and share common signalling mechanisms based on the common usage of a group of cytosolic non-receptor tyrosine kinases of the Janus kinase (JAK) family [1,6]. In the case of the GHR, JAK2 is recruited to Box1 after one GH molecule interacts with two identical GHR molecules. This binding results in the activation of the JAK2 molecules and subsequent tyrosine phosphorylation of JAK2, GHR and signal transduction and activators of transcription (STATs). This leads to a cascade of events initiating signal transduction. In addition, GH induces the activation of the mitogen-activated protein kinase ('MAP kinase') and the insulin receptor substrate ('IRS') pathway [7].

GH action depends not only on the presence of the hormone in the circulation, but also on the availability of its receptor at the

cell surface of the target cells. The maintenance of GH binding capacity depends, on one hand, on the synthesis of new receptor in the endoplasmic reticulum (ER), receptor processing in the Golgi and transport to the plasma membrane and, on the other hand, the removal of GHR from the cell surface. Two processes are known to contribute to receptor turnover: (i) proteolysis at the cell surface results in the release of the GH-binding protein ('GHBP'), a process referred to as shedding [8], whereas (ii) receptor endocytosis leads to the removal of the receptor molecule from the cell surface, followed by degradation in the lysosome. Shedding of the GH-binding protein involves the action of the tumour necrosis factor- α -converting enzyme ('TACE'), a transmembrane metalloprotease [9]. Ectodomain shedding of the GHR can be influenced by phorbol esters and growth factors [10].

Endocytosis of the GHR is regulated by the ubiquitin–proteasome system [11], a system best known for its role in the degradation of short-lived and abnormal soluble proteins in the cytosol [12]. Recent studies [13–15] have identified a role of ubiquitination in endocytosis. Ligand binding results in increased ubiquitination of several mammalian receptor proteins, such as epidermal growth factor ('EGF') receptor [16], platelet-derived growth factor ('PDGF') receptor [17], Met receptor [18], *c-kit* receptor [19], T-cell receptor [20] and GHR [21]. C-cbl, a tyrosine kinase adaptor protein, is an E3 ubiquitin ligase that mediates epidermal growth factor receptor ubiquitination and regulates sorting of the receptor into multivesicular bodies, thereby attenuating kinase signalling [22]. For the Met receptor, recent evidence [23] suggests that degradation occurs mainly

Abbreviations used: 2D, two-dimensional; CHO, Chinese-hamster ovary; DTT, dithiothreitol; ER, endoplasmic reticulum; F327A, mutation of Phe³²⁷ → Ala; GH, growth hormone; GHR, GH receptor; GST, glutathione S-transferase; Hsp, heat-shock protein; Hsc, heat-shock cognate protein; CHIP, C-terminus of Hsc70-interacting protein; Hop, Hsc70/Hsp90-organizing protein; JAK, Janus kinase; MEM, minimal essential medium; NTA, nitrilotriacetate; PPR, pentatricopeptide repeat; TPR, tetratricopeptide repeat; SGT, small glutamine-rich TPR-containing protein; hSGT, human SGT; UbE, ubiquitin-dependent endocytosis; wt, wild-type.

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in lysosomes and that proteasome inhibitors interfere with endocytic trafficking. However, only for the GHR has it been shown that endocytosis is mediated by the ubiquitin–proteasome system via a 10 amino acid motif within the GHR cytosolic tail [ubiquitin-dependent endocytosis (UbE) motif; Asp-Ser-Trp-Val-Glu-Phe-Ile-Glu-Leu-Asp] [24]. Mutations of several amino acids within the motif, such as Phe³²⁷ → Ala (F327A), abolishes ubiquitination as well as internalization. The UbE motif can thus be considered a specific target for the ubiquitin system.

In the present study, we searched for proteins that specifically target the UbE motif and investigated whether they are involved in the ubiquitination and internalization of the GHR. Using GST fusion proteins encompassing the UbE motif, we pulled down a 35 kDa protein, identified by MS as small glutamine-rich tetratricopeptide repeat (TPR)-containing protein (SGT). SGT, also known as viral protein U-binding protein (Ubp), was originally discovered as a protein interacting with envelope proteins of two viruses, namely parvovirus H-1 and HIV type 1 [25,26]. Sequence analysis of SGT revealed three tandem TPRs. TPR motifs are highly degenerated 34 amino acid repeats involved in protein–protein interaction [27]. TPR-containing proteins show E3 ligase activity, such as the C-terminus of heat-shock cognate protein 70 (Hsc70)-interacting protein (CHIP) [28], or are able to interact with subunits of the SCF (Skp1/cullin/F box) complex, such as sgt1 [29]. For SGT, it has been shown that the TPR motif is responsible for the interaction with the C-terminal domain of Hsc70 [30]. In an *in vitro* study, SGT negatively influenced the ability of Hsc70 and DnaJ to refold a denatured model protein substrate [31]. Recent studies [32] also indicated a role of SGT in a trimeric protein complex that functions as a synaptic chaperone machinery. SGT was also identified to interact with β -amyloid peptide. Double-stranded RNA inhibition results in suppression of toxicity associated with β -amyloid expression [33]. Our present results show that SGT is a GHR-interacting protein both *in vitro* and *in vivo* and is interacting UbE motif specific. The activity of the ubiquitin system plays no role in the interaction between SGT and the GHR. SGT binds to the precursor as well as the mature form of the receptor and interacts specifically via its first TPR motif with the UbE motif of the GHR.

EXPERIMENTAL

Materials

phSGT/15b expressing human SGT (hSGT) with an N-terminal His-tag in Pet-15b was a gift from Dr Chung Wang (Institute of Molecular Biology, Academia Sinica, Taipei). phSGT/15b was expressed in *Escherichia coli* (strain BL21) and His-tagged hSGT was purified by Ni²⁺-nitrilotriacetate (NTA)-agarose beads (Qiagen) according to the manufacturer's instructions. Full-length rabbit GHR cDNA in pCB6 was as described previously [21]. Truncated GHR constructs, GHR334 and GHR434, were constructed by introducing a stop codon at the proper positions in the GHR cDNA and were subcloned into pcDNA3.1 (Invitrogen) as described previously [11]. cDNA of internalization-deficient mutants GHR^{F327A} and GHR334^{F327A} were constructed by site-directed mutagenesis as described previously [11]. An antibody (Mab5) directed against the extracellular domain of the GHR was from AGEN Inc. (Parsippany, NJ, U.S.A.). An antibody recognizing hSGT was generated against His-tagged hSGT. Culture media, fetal calf serum (FCS), L-glutamine and antibiotics for tissue culture were purchased from Gibco.

Production of GHR–GST fusion proteins

Full-length GHR cDNA in pCB6 served as template for the PCR reaction. GHR(271–334) was amplified with a 5'-oligonucleotide introducing a *Bam*HI restriction site before amino acid 271 and a 3'-oligonucleotide introducing a stop codon after amino acid 334 followed by a *Bam*HI restriction site. For amplification of GHR(271–334)^{F327A}, PCR reactions were performed with the same 5'-oligonucleotide and a 3'-oligonucleotide that introduced the F327A mutation, a stop codon after amino acid 334 followed by a *Bam*HI site. The PCR fragments were cloned into pGEX-3X (Amersham Biosciences) using the *Bam*HI restriction enzyme sites. The identity of the DNA constructs was confirmed by DNA sequencing. The resulting plasmid was used to express GST–GHR(271–334) and GST–GHR(271–334)^{F327A} in *E. coli* (strain BL21). The synthesis of recombinant proteins was induced by isopropyl β -D-thiogalactoside. GST and GST fusion proteins were purified with GSH–Sepharosebeads (Amersham Biosciences) by the procedure recommended by the manufacturer.

Production of His-tagged hSGT

phSGT/15b was used to express hSGT in *E. coli* (strain BL21). The synthesis of recombinant proteins was induced by isopropyl β -D-thiogalactoside. hSGT was isolated using Ni²⁺-NTA-agarose beads (Qiagen) by the procedure recommended by the manufacturer.

Generation of hSGT and hSGT partial constructs

phSGT/15b served as template for the PCR reaction. hSGT full-length construct was generated with a 5'-oligonucleotide introducing a *Bam*HI restriction site before amino acid 2 and a 3'-oligonucleotide introducing a *Bam*HI restriction site after the stop codon. The partial constructs hSGT192, hSGT158 and hSGT124 were generated with the same 5'-oligonucleotide as full-length hSGT. The 3'-primers introduced a stop codon after amino acid 192, 158 and 124 respectively, and an *Apa*I restriction site after the stop codon. For constructs hSGT Δ 90, hSGT Δ 124 and hSGT Δ 192, the same 3'-oligonucleotide was used, which introduced an *Eco*RV restriction site after the stop codon of SGT. The 5'-oligonucleotides introduced a *Bam*HI site and started annealing at amino acid 91, 125 and 193 respectively. The PCR fragments were cloned into pGEM-T easy (Promega) for sequencing. The inserts were cloned into pcDNA3.1/HisB (Invitrogen) or pEF6/HisB (Invitrogen) using the restriction sites introduced by the oligonucleotides.

Mammalian cells and transfection

HepG2 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FCS, 10 units/ml penicillin and 100 mg/ml streptomycin.

Chinese-hamster ovary (CHO) ts20 cells, bearing a thermolabile ubiquitin-activating enzyme E1 that is inactive at the non-permissive temperature of 41.5 °C, were used. CHO ts20 cells, stably expressing various GHR constructs, were propagated at 30 °C with minimal essential medium (MEM) α supplemented with 4.5 g/l glucose, 10 % (v/v) FCS, 10 units/ml penicillin, 100 mg/ml streptomycin and 0.45 mg/ml geneticin. For experiments, cells were grown in 60-mm dishes in the absence of geneticin to a confluency of approx. 80 %. Cells received 10 mM sodium butyrate 16 h before experiments were performed to enhance expression of cytomegalovirus-driven constructs.

CHO E36 cells were propagated at 30 °C with MEM α supplemented with 4.5 g/l glucose, 10 % (v/v) FCS, 10 units/ml penicillin and 100 μ g/ml streptomycin.

For transient transfection, CHO ts20 and E36 cells were grown to 30 % confluency and transfected with GHR334 and GHR334^{F327A} DNA using the calcium phosphate method. At 24 h after transfection, the cells were treated with 10 mM sodium butyrate overnight. Cells were used for experiments 36 h after transfection.

Metabolic labelling and subcellular fractionation

For metabolic labelling, HepG2 cells were incubated in methionine-free MEM α before the addition of [³⁵S]methionine (3.7 MBq/ml Tran [³⁵S] label, 40 TBq/mmol; ICN Biochemicals) for 2 h. After incubation for 5 min with MEM α , cells were swollen in LS buffer [10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol (DTT)] containing protease inhibitors (1 mg/ml leupeptin, 1 mg/ml pepstatin and 1 mM PMSF) for 15 min. Cells were broken by passing through a 30-gauge needle. Postnuclear supernatants were prepared by centrifugation of homogenates for 5 min at 320 g at 4 °C. High-speed supernatants and membrane pellets were obtained by centrifugation of postnuclear supernatants at 65 000 g for 1 h at 4 °C in an ultracentrifuge. BSA and Triton X-100 were added to a final concentration of 0.5 % (w/v) to the supernatant.

In vitro transcription translation

hSGT cDNA or cDNA of SGT partial constructs in pcDNA3.1/HisB was used in an *in vitro* transcription/translation system supplemented with 3.7 MBq/ml [³⁵S]methionine and TNT[®]-T7-coupled reticulocyte lysate ready reaction mix, according to the manufacturer's instructions (Promega).

In vitro binding assay

A portion (200 μ l) of the cytosolic fraction or 7.5 μ l of *in vitro* transcription/translation mixture (³⁵S-labelled hSGT truncations) was incubated for 1 h at 4 °C with GST, GST-GHR(271–334) or GST-GHR(271–334)^{F327A} absorbed on to GSH-Sepharose beads in 200 μ l of homogenization buffer [LS buffer containing 0.5 % (w/v) Triton X-100, 0.5 % (w/v) BSA and protease inhibitors]. Beads were pelleted in an Eppendorf centrifuge and washed three times with 1 ml of homogenization buffer, and washed twice with PBS containing protease inhibitors. The beads were boiled in 2 $\times$ sample buffer. Samples were subjected to SDS/PAGE or two-dimensional (2D) gel electrophoresis. Proteins associated with the GST fusion proteins were visualized by autoradiography of the dried gel or by Coomassie Blue staining. Autoradiograms were quantified using Imagequant software (Molecular Dynamics).

To investigate direct interaction between SGT and GHR, we used 10 μ g of purified GST-GHR fusion proteins and 10 μ g of purified hSGT in 200 μ l of a 50 % slurry of GSH-Sepharose beads and 200 μ l of homogenization buffer. The same procedure as described above was used.

2D gel electrophoresis

The proteins from the *in vitro* binding experiments were resuspended in 350 μ l of buffer containing 6 M urea, 2 M

thiourea, 10 mM DTT, 2 % (w/v) CHAPS, 1 % (v/v) Resolytes pH 3.5–10 (BDH), 1 % (v/v) Resolytes pH 4–8 (BDH) and Bromophenol Blue. For the isoelectric focusing, non-linear 18 cm Immobiline Dry Strips, pH 3–10, (Amersham Biosciences) were used. Rehydration of the strips with 320 μ l of protein samples was performed overnight at room temperature. A MultiphorII Electrophoretic Unit (Amersham Biosciences) was used for isoelectric focusing, which was performed at 15 °C starting with 300 V. The voltage was increased to 3500 V within 3 h and continued at 5000 V increments until 90 000 V were reached. The strips were incubated under reducing conditions [6 M urea, 2 % (w/v) SDS, 30 % (v/v) glycerol, 25 mM Tris/HCl (pH 8.8) and 2 % (w/v) DTT] for 20 min, and the reaction was stopped for 20 min in an iodoacetamide solution [6 M urea, 2 % (w/v) SDS, 30 % (v/v) glycerol, 25 mM Tris/HCl (pH 8.8) and 2.5 % (w/v) iodoacetamide]. Proteins were separated on a 9–16 % gradient gel using a Protean II Multi Cell Chamber (Bio-Rad). Proteins were stained with Coomassie Blue, and protein spots were excised and subjected to MS.

MS

The protein gel spots of interest were treated with DTT (10 mM in 100 mM NH₄HCO₃) and iodoacetamide (55 mM in 100 mM NH₄HCO₃) and in-gel digested as described by Shevchenko et al. [34]. The tryptic peptides obtained were analysed by nanoscale LC-MS [35]. Briefly, peptide mixtures were loaded on to a 50 μ m (internal diameter) $\times$ 42 mm precolumn packed with 10 μ m C8 particles (Poros 10R1; Perseptive). After loading, the trapped peptides were separated on a 50 μ m (internal diameter) $\times$ 206 mm length column packed with 5 μ m C18 particles (Purosphere) and, subsequently, eluted into an ion-trap mass spectrometer (LCQ, Finnigan). Fragment ion spectra were recorded in data-dependent acquisition mode.

Cell lysis, immunoprecipitation and Western blotting

Cells were grown in 60-mm dishes to a confluency of 90 % and lysed on ice in 300 μ l of homogenization buffer. The lysates were cleared by centrifugation. Immunoprecipitation of the supernatant was carried out with anti-GHR or -SGT antibodies for 2 h on ice in homogenization buffer. Protein A-agarose beads (Repligen Co., Cambridge, MA, U.S.A.) were used to isolate the immunocomplexes. The immunocomplexes were washed three times with homogenization buffer, and washed twice with PBS. Immunocomplexes were analysed by SDS/PAGE [4–20 % (w/v) gels], together with total cellular lysate, and transferred on to a PVDF membrane. The blots were immunostained with either anti-GHR or anti-SGT antibodies. After incubating the blots with Protein A conjugated to horseradish peroxidase, antigen-antibody complexes were visualized using the enhanced chemiluminescence system (Boehringer Mannheim).

RESULTS

Identification of binding partners for the UbE motif of GHR

To search for potential binding partners for the UbE motif of the GHR, which are involved in the internalization step, we performed GST-pulldown experiments. GST or GST fusion proteins, encompassing the wild-type (wt) UbE motif [GST-GHR(271–334)] or the inactive motif [GST-GHR(271–334)^{F327A}] (Figure 1A), were incubated with the cytosolic fraction of

Figure 1 Identification of binding partners for the UbE motif of GHR

(A) Schematic representation of the wtGHR, GST–GHR(271–334) and GST–GHR(271–334)^{F327A} fusion proteins used in the pull-down experiments. TM represents the transmembrane domain, and the location of the UbE motif in the constructs is shown. A, the location of the alanine residue in the GST–GHR(271–334)^{F327A} construct. (B) HepG2 cells were labelled for 2 h with [³⁵S]methionine and solubilized in homogenization buffer. Equal amounts of GST or GST fusion proteins were immobilized on GSH–Sepharose beads and incubated with equal amounts of cytosolic fraction for 1.5 h. Beads were washed with homogenization buffer before subjecting the samples to SDS/PAGE. (C) Samples were prepared as described in (B) and subjected to 2D gel electrophoresis. * indicates the specific 35 kDa band identified. Molecular-mass markers (in kDa) are indicated on the left.

HepG2 cells metabolically labelled with [³⁵S]methionine. The autoradiogram showed a predominant 35 kDa protein band that was specifically isolated by the GSH–Sepharose beads carrying the UbE motif of GHR, but not by those with GST or the inactive UbE motif (Figure 1B). In addition, two stronger specific bands of 130 kDa and 150 kDa were visible. Because the 35 kDa band showed such a strong interaction, we decided to concentrate on this band. We scaled up the purification protocol to obtain Coomassie Blue-stainable amounts of the 35 kDa protein. 2D gel electrophoresis was used to separate the 35 kDa protein from the GST fusion proteins, which migrated at approximately the same position in the gel. The 35 kDa protein migrated as four individual spots with a pI ranging from 4.4–4.9 (Figure 1C). All four protein spots were subjected to microsequencing by MS and were identified as SGT. SGT was first identified as a protein putatively interacting with envelope proteins of two viruses, i.e. HIV type 1 and parvovirus H-1.

In order to investigate whether SGT interacted directly with GHR *in vitro*, equal amounts of His-tagged SGT expressed in bacteria and purified with Ni²⁺-NTA-agarose beads were incubated with GST, GST–GHR(271–334) or GST–GHR(271–334)^{F327A} immobilized on GSH–Sepharose beads. This experiment

was performed in the absence or presence of HepG2 cell lysate. As shown in Figure 2, SGT specifically interacted with GST–GHR(271–334), the fusion protein containing the intact UbE motif. No interaction was observed with GST or GST–GHR(271–334)^{F327A}. This indicates that the interaction between SGT and the UbE motif of the GHR is direct and specific for the UbE motif. Addition of HepG2 lysate did not result in a significant increase in the interaction between SGT and GST–GHR(271–334).

Identification of the domain of SGT that is involved in the GHR–SGT interaction

In order to map the UbE motif-interacting domain of SGT, we constructed several partial constructs of SGT (Figure 3A). *In vitro* translated ³⁵S-labelled SGT constructs were incubated with purified GST, GST–GHR(271–334) or GST–GHR(271–334)^{F327A} bound to GSH–Sepharose beads. The expression of the SGT constructs was confirmed (results not shown). As expected, full-length SGT showed strong interaction with GST–GHR(271–334), which was 30 times stronger than the interaction with

Figure 2 *In vitro* interaction of GHR with SGT

GST–GHR fusion proteins and His-tagged SGT expressed in bacteria were incubated in the absence or the presence of HepG2 lysate for 1.5 h. Equal amounts of GST or GST fusion proteins were immobilized on GSH–Sepharose beads and the beads were washed with homogenization buffer before subjecting the samples to SDS/PAGE. The gel was stained with Coomassie Blue.

GST–GHR(271–334)^{F327A} or GST alone (Figures 3B and 3C). Deletion of the C-terminus together with TPR2 and TPR3 (SGT124) resulted in a decrease in the interaction between GST–GHR344 and hSGT; however, this interaction was still dependent upon an intact UbE motif. A decreased, but specific, interaction that still depended on the UbE motif was also observed with SGT158 and SGT192. Both constructs lack the C-terminus (SGT192) and, in the case of SGT158, also TPR3. Removing the N-terminus (SGTΔ90) flanking the TPR motifs drastically increased the interaction by approx. 3-fold compared with the full-length hSGT construct. However, the C-terminus alone (SGTΔ192) was not able to interact with the UbE motif of the GHR. In addition, construct SGTΔ124 was not able to interact with the GHR UbE motif *in vitro*. The domain that all SGT motifs showing an UbE motif-dependent interaction with the GST–GHR fusion proteins had in common was TPR1, demonstrating that TPR1 is the domain of SGT that is involved in the interaction with the GHR.

Characterization of GHR–SGT interaction *in vivo*

We used CHO ts20 cells stably expressing wtGHR, GHR434, GHR334 or their corresponding F327A mutant to confirm this GHR–SGT interaction *in vivo* (Figure 4A). The cytosolic tail of GHR334 is comparable with the part of the cytosolic tail of GHR that was fused to GST. Immunoprecipitations using an antibody raised against full-length SGT resulted in co-immunoprecipitation of the wtGHR as well as of the truncated GHRs. In all cases, both the precursor and the mature form of the receptor were immunoprecipitated (Figure 4B). In the case of the wtGHR, less receptor was immunoprecipitated compared with the truncated receptors. If cell lines expressing the respective mutant receptors were used, no co-immunoprecipitation was observed. The band visible at 55 kDa after co-immunoprecipitation of GHR334^{F327A} does not represent the precursor of the GHR, but rather IgG, which was also recognized by the anti-GHR antibody. This band is also visible in the GHR434 co-immunoprecipitation. For this GHR truncation, the precursor form runs at 75 kDa. These results suggest that the interaction of SGT is independent of the length of the cytosolic tail of the GHR and that SGT interacts with both the precursor and the mature form of the receptor *in vivo*.

Involvement of the ubiquitin system in the SGT–GHR interaction

As the UbE motif is required for ubiquitin–proteasome system-dependent internalization of the GHR, we investigated whether the ubiquitin system plays a role in the interaction between SGT and GHR. We used the CHO ts20 cell line containing a thermolabile ubiquitin-activating enzyme, E1, which is inactive at the non-permissive temperature of 41.5 °C. As a control cell line, CHO E36 cells were used. Transient transfection of both cell lines with either GHR334 or GHR334^{F327A} resulted in the expression of similar amounts of GHR in both cell lines (Figure 5, upper panel). Because of the transient transfection, the precursor form of the receptor was predominantly produced. After incubating the cells at the permissive or non-permissive temperature, GHR was co-immunoprecipitated with the anti-SGT antibody. At 41.5 °C the amount of receptor had increased, due to the block of receptor internalization and subsequent degradation. Inactivation of the ubiquitin system did not influence the interaction of SGT with GHR (Figure 5, lower panel). Due to the accumulation of receptor at 41.5 °C, the amount of receptor interacting with SGT was increased. These results suggest that the interaction between SGT and the GHR is independent of an intact ubiquitin system and/or that the ubiquitin system acts downstream of SGT.

DISCUSSION

In the present study, we identified a potential downstream effector of GHR function called SGT. SGT is able to bind specifically to the UbE motif of the GHR *in vitro* and *in vivo*. Four different species of SGT, which differ in pI, were identified in pulldown experiments with the UbE motif of the GHR. Putative myristoylation, glycosylation and phosphorylation sites have been postulated for SGT [25]. Regarding the small difference in the molecular mass of the isolated SGT species, it is very unlikely that these SGT species are myristoylated or glycosylated. Instead, phosphorylation would be the most likely explanation for the difference in pI. It has been shown for other TPR-containing proteins that phosphorylation may represent a mechanism by which the interaction with particular proteins can be modulated: Miyata et al. [36] have demonstrated that phosphorylation of FK506-binding protein 52 ('FKBP52'), another TPR-containing protein, by casein kinase II affects the interaction of this protein with heat-shock protein 90 (Hsp90). Phosphorylation and dephosphorylation might be involved in the regulation of the SGT–GHR interaction but, as the four SGT species have approximately the same intensity, binding of SGT to the GHR is probably not dependent on this modification. Whether this modification, which does not represent tyrosine phosphorylation (results not shown), plays a role in the interaction with GHR remains to be established. Because SGT contains three TPR motifs, it is tempting to speculate that these modifications occur in the TPR motifs themselves.

The interaction between the UbE motif of the GHR and SGT takes place through the first TPR motif of SGT, as no interaction with the GHR UbE motif was visible with hSGTΔ124, the construct lacking the first TPR motif (TPR1). The C-terminus of SGT is able to strengthen this interaction in the absence of the N-terminus of SGT. However, the presence of TPR1 is essential for this interaction, demonstrating that the C-terminus might play a role in this interaction, but it is not sufficient. The two remaining TPR motifs were not able to substitute for the missing TPR motif of SGT, indicating that they might be involved in other protein interactions. Deletion of the first TPR motif may also

Figure 3 Identification of the domain of SGT that is involved in the GHR-SGT interaction

(A) Schematic representation of SGT and partial constructs of SGT truncations used in the GST-pulldown experiments. The location of the single TPR motifs (TPR1–3) are shown. (B) Equal amounts of GST or GST fusion proteins were immobilized on GSH-Sepharose beads and incubated with equal amounts of *in vitro* transcription mix for 1.5 h. Samples were subjected to SDS/PAGE and autoradiography. Molecular-mass markers (in kDa) are indicated on the left. (C) The autoradiogram in (B) was quantified by phosphorimaging. Closed bars, GST-GHR334; grey bars, GST-GHR334^{F327A}. The intensity of GST was subtracted from the intensity of the GST fusion protein.

result in the disturbance of the structure of the TPR domain and thereby prevent the interaction between GHR and SGT. For the interaction of SGT with Hsc70, it has been demonstrated [30] that partial removal of the first and second TPR motifs resulted in a loss of interaction in a yeast two-hybrid system. Scheufler et al. [37] showed that conserved residues of all three TPR motifs of each of the TPR domains of Hsc70/Hsp90-organizing protein (Hop) contribute to the interaction with Hsp70 or Hsp90. The TPR1 domain of Hop recognizes the C-terminal sequence Thr-Ile-Glu-Glu-Val-Asp of Hsp70. The C-terminal aspartate residue of Hsp70 forms a two-carboxylate clamp with the highly conserved residues of the TPR domain, which is responsible for a tight interaction between the Hsp and Hop. A similar interaction is possible in the case of the GHR334 truncation, which possesses a C-terminal aspartate residue, and SGT and could therefore form a two-carboxylate clamp with the TPR domain of SGT. However, in the case of full-length GHR, which possesses a C-terminal

proline residue rather than an aspartate residue, the interaction still occurs. Furthermore, the interaction depends upon the UbE motif of the GHR and mutation of F327A in the GHR abrogates the interaction with SGT. Alignment of the UbE motif with the C-terminus of Hsp70 shows that the position of the phenylalanine residue in the UbE motif of the GHR corresponds with the position of the glycine residue in the C-terminus of Hsp70. Mutation of the glycine residue to alanine hardly influences the interaction of Hsp70 with Hop, whereas the interaction of the GHR with SGT depends on the presence of the phenylalanine residue at this position. Therefore we can conclude that the formation of a two-carboxylate clamp between the GHR and SGT is not very likely and that different amino acids than in the case of the interaction between Hsp70 and Hop are involved in this interaction. Although it cannot be excluded that the interaction occurs through Hsp70, this is unlikely because the first TPR motif of SGT alone is not sufficient to bind to Hsc70, but SGT

Figure 4 *In vivo* characterization of the GHR-SGT interaction

(A) Schematic representation of the GHR truncations used, with the transmembrane (TM) domain and the UbE motif indicated. A, the location of the alanine residue in the GST-GHR(271-334)^{F327A} construct. (B) CHO cells stably expressing either wtGHR, GHR^{F327A} or the respective truncations at amino acid 334 and 434 were lysed in homogenization buffer. SGT was immunoprecipitated (IP) from the cell lysates by the addition of an anti-SGT antibody and samples, together with total cellular lysates (Direct lysate), were analysed by SDS/PAGE and Western blotting (WB) with an anti-GHR antibody (Mab5). Molecular-mass markers (in kDa) are indicated on the left.

constructs containing the first TPR motif only can interact with the UbE motif of the GHR. Furthermore, we have identified another protein, leucine-rich protein of 130 kDa ('LRP130'), that contains nine pentatricopeptide repeat (PPR) motifs [38] as an UbE motif-binding protein (J. A. Schantl and G. J. Strous, unpublished work). PPR motifs show high sequence similarity with TPR motifs and are predicted to create extended superhelical structures involved in protein-protein interactions [39]. PPR-containing proteins are abundant in *Arabidopsis thaliana*, where they localize to mitochondria and peroxisomes. Functionally several of these proteins have been implicated in RNA processing [39].

There are several possibilities for SGT action in GHR function. SGT might function in the process of quality control in the ER, be involved in the transport from the ER to the plasma membrane or be connected to the transport of the receptor from the plasma membrane to the lysosome. From our present results, it is clear that SGT can interact with the precursor as well as with the mature form of the GHR. This fact also gives some clues as to where the interaction between the GHR and SGT might take place. The precursor form of the receptor resides in the ER from where it is transported to the Golgi and becomes complex glycosylated. As we can already detect the precursor form of the GHR interacting with SGT, it is quite possible that the interaction already takes place in the ER, possibly as early as during translation of the

Figure 5 Involvement of the ubiquitin system in SGT-GHR interaction

CHO ts20 cells, containing a thermolabile ubiquitin-activating enzyme E1, or CHO E36 cells were transfected with either GHR334 or GHR334^{F327A}. Cells were incubated for 1 h at 30 °C or 41.5 °C and cell lysates were prepared. Upper panel, lysates were analysed by Western blotting (WB) with an anti-GHR antibody (Mab5). p and m indicates the precursor (110 kDa) and mature GHR (130 kDa) respectively. Lower panel, SGT was immunoprecipitated (IP) from the cell lysates by the addition of an anti-SGT antibody and samples were analysed by SDS/PAGE and Western blotting (WB) with an anti-GHR antibody (Mab5). Molecular-mass markers (in kDa) are indicated on the left.

protein. This interaction could play a role in the correct folding of the GHR as well as in the process of receptor dimerization, which has been shown to occur already in the ER [40]. However, this is unlikely, because there is no defective dimerization in the GHR^{F327A} mutant. The latter does not exclude the possibility that SGT might recognize monomeric receptor molecules and is involved in the removal of monomeric receptors from the ER or maybe from the plasma membrane if they were able to escape quality control in the ER.

Concerning the possible involvement of SGT in the transport of GHR from the ER to the plasma membrane, we observed by immunofluorescence that overexpression of a SGT construct lacking the N- or C-terminus of SGT, or the construct missing the N-terminus together with TPR1 did not show an effect on the distribution of the GHR (results not shown). Due to the presence of endogenous SGT, we cannot exclude that overexpression of the partial constructs was not high enough to induce a dominant-negative effect.

When we examined the effect of overexpressing putative dominant-negative constructs on the uptake of GH, we did not observe any effect on the internalization of the GHR-GH complex. Again the dominant-negative effect might not be visible, due to the high endogenous level of SGT. SGT could play a role in a later step of endocytosis.

The ubiquitin system, which is involved in the internalization of the GHR, does not affect the interaction of SGT with the UbE motif. This indicates that the GHR-SGT interaction might precede the action of the ubiquitin system. At present we cannot exclude the possibility that SGT is interacting with the UbE motif until the ubiquitin system is triggered to interact with the receptor. Therefore SGT might prevent the interaction of the ubiquitin machinery with the receptor on its way from the ER to the cell surface. Future experiments using small RNA-interference might be the method of choice to examine this.

Finally, there is a possible role for SGT in the sorting of the GHR from the endosome to lysosome. This sorting process depends both on an intact UbE motif [41] and the action of the

proteasome [42]. As an intact UbE motif is also a prerequisite for SGT interaction, the function of SGT in sorting from endosomes to the lysosome cannot be excluded.

The TPR motifs within SGT might give an indication to its function. TPR motifs have been identified in more than 50 functionally unrelated proteins present in organisms as diverse as bacteria and humans. Most TPR-containing proteins are associated with multi-protein complexes, and there is extensive evidence demonstrating that TPR motifs are important in the function of chaperones, cell cycle, transcription and protein transport complexes. All reports about SGT and a putative function of SGT link the protein to chaperone complexes. Its ability to interact with Hsc70 and to negatively influence the capacity of Hsc70 together with DnaJ to refold an unfolded substrate indicates a role as a negative regulator of Hsc70 function. For example, CHIP is a co-chaperone that converts Hsc70 from a protein-folding machine into a degradation factor that functions at the level of the ER. Unlike SGT, CHIP shows E3 ligase activity, which is associated with a U-box at the C-terminus of the protein. In addition, Imai et al. [43] reported recently that CHIP can interact directly with Parkin, a RING E3 ligase. By interacting with Parkin, CHIP replaces Hsc70 that can bind to the same region of Parkin and, at the same time, promotes the ubiquitination activity of Parkin. Another TPR-containing protein, sgt1, was identified because of its ability to interact with skp1, a subunit of the SCF E3 ligase complex [29]. For SGT, there is no sequence similarity to either a U-box sequence or any other sequence that might hint at a function of SGT in ubiquitin ligation. Nevertheless, it is tempting to speculate that SGT is able to interact with components of the ubiquitin–proteasome system and thereby links the GHR to the ubiquitin–proteasome system. Taken together, SGT might act as a modulator of ubiquitin system controlled down-regulation of the GHR by competing for the UbE motif.

We would like to thank Hans Dieplinger and Barbara Schantl for help with 2D electrophoresis, and Jürgen Gent, Peter van Kerkhof and Martin Sachse for stimulating discussions. This work was supported by grants of the Netherlands Organization for Scientific Research (NWO-902-23-192), and a European Union Network grant (ERBFMRXCT96-0026).

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Received 10 October 2002/23 April 2003; accepted 8 May 2003

Published as BJ Immediate Publication 8 May 2003, DOI 10.1042/BJ20021591