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FBW2 Targets GCMa to the Ubiquitin-Proteasome Degradation System*

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The GCM proteins GCMa/1 and GCMb/2 are novel zinc-containing transcription factors critical for glial cell differentiation in fly and for placental as well as parathyroid gland development in mouse. Previous pulse-chase experiments have demonstrated differential protein stabilities of GCM proteins with half-lives from ~30 min to 2 h (Tuerk, E. E., Schreiber, J., and Wegner, M. (2000) *J. Biol. Chem.* 275, 4774–4782). However, little is known about the machinery that controls GCM protein degradation. Here, we report the identification of an SCF complex as the GCM ubiquitin-protein isopeptide ligase (E3) that regulates human GCMa (hGCMa) degradation. We found that SKP1 and CUL1, two key components of the SCF complex, associate with hGCMa *in vivo*. We further identify the human F-box protein FBW2 (hFBW2) as the substrate recognition subunit in the SCF E3 complex for hGCMa. We show that hFBW2 interacts with hGCMa in a phosphorylation-dependent manner and promotes hGCMa ubiquitination. Supporting a critical role for hFBW2 in hGCMa degradation, knockdown of h*FBW2* expression by RNA interference leads to a reduction in hGCMa ubiquitination and a concomitant increase in hGCMa protein stability. Our study identifies the SCF^{hFBW2} E3 complex as the key machinery that targets hGCMa to the ubiquitin-proteasome degradation system.

The *gcm1* (*glial cell missing 1*) gene was first identified in a *Drosophila melanogaster* mutant line that produces additional neurons at the expense of glial cells. Conversely, ectopic expression of *gcm1* leads to glial differentiation at the expense of other cell types (1, 2). In *D. melanogaster*, GCM1 mediates the differentiation of lateral (but not midline) glial cells and the proliferation of plasmatocyte precursors (1, 2). Recently, another *gcm* gene in *D. melanogaster*, *gcm2*, was identified and shown to have biological functions similar to those of *gcm1* (3, 4). Two homologs of *Drosophila gcm1* named *GCMa* and *GCMb* have also been isolated from mouse and human (5, 6). Unlike the *Drosophila gcm* genes, the mammalian *GCM* genes are not primarily expressed in the nervous system. Mouse *GCMa* is highly expressed in labyrinthine trophoblast cells (7). The GCMa knockout mouse is embryonic lethal, with a failure to form a labyrinth layer and no fusion of trophoblast cells to syncytiotrophoblasts (8, 9). In contrast, mouse *GCMb* is expressed in parathyroid cells, and the GCMb knockout mouse fails to develop a parathyroid gland (10). Therefore, although *Drosophila gcm* and mammalian *GCM* genes are evolutionarily conserved at the nucleotide and protein levels, they appear to execute different biological functions during development.

GCM proteins form a novel family of transcription factors that all share sequence homology in the N-terminal region that constitutes the DNA-binding domain called the GCM motif, which has a preferred binding sequence of 5′-(A/G)CCC(T/G)CAT-3′ or its complement (11, 12). Moreover, two zinc ions have been found to be tightly coordinated by cysteine and histidine residues and to be required for the DNA-binding activity in the GCM motif (13). Sequence homology is less preserved outside the GCM motif; a transactivation domain has been identified in the C terminus of GCM proteins (11, 14). Target genes of fly GCM1 include *gcm1* and several glial cells-specific genes such as *pointed*, *repo*, *tramtrack*, and *prospero* (15). Target genes of human (h)[1] GCMa have been reported recently. For instance, the promoter region of a placental aromatase gene that is responsible for estrogen biosynthesis has been found to contain GCM-binding sites that are recognized by GCMa (16). In addition, we have demonstrated that hGCMa regulates expression of the syncytin gene, which encodes a fusogenic membrane protein mediating the fusion of trophoblast cells (17). Therefore, hGCMa appears to be a key factor in the formation of the human syncytiotrophoblast layer.

GCM proteins contain PEST sequences, which are frequently found in labile proteins. Previous pulse-chase experiments have shown that GCM proteins have short half-lives from ~30 min to 2 h (18). Because GCM proteins are labile and because little is known about the machinery that controls GCM protein degradation, in this study, we investigate the role of the ubiquitin-proteasome degradation system in GCM protein turnover.

Ubiquitin-mediated protein degradation plays an important role in controlling numerous processes, including cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis. Ubiquitin is a polypeptide of 76 amino acids, and its primary sequence is highly conserved from yeast to mammals. Ubiquitin conjuga-

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[1] The abbreviations used are: h, human; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; RNAi, RNA interference; HA, hemagglutinin; mAb, monoclonal antibody; Ub, ubiquitin; λ-PPase, λ-protein phosphatase; GST, glutathione *S*-transferase; siRNA, small interfering RNA.

tion of target proteins requires a cascade of reactions. First, ubiquitin is activated by the ubiquitin-activating enzyme in an ATP-dependent process. Second, activated ubiquitin is transferred to the ubiquitin-conjugating enzyme (ubiquitin carrier protein (E2)). Finally, E2 transfers ubiquitin to a substrate protein by itself or in cooperation with ubiquitin-protein isopeptide ligase (E3) by formation of an amide isopeptide bond between the carboxyl group of the C-terminal glycine residue of ubiquitin and the ϵ-amino group of an internal lysine residue of the substrate protein (19). The monoubiquitinated substrate protein becomes polyubiquitinated via the same cascade of reactions. In eukaryotes, polyubiquitinated proteins are targeted to the 26 S proteasome, which consists of a proteolytic core particle, the 20 S proteasome, and two 19 S regulatory complexes (20).

In the ubiquitin-proteasome degradation system, E3 controls substrate specificity and transfers ubiquitin to lysine residues of the substrate protein. E3 ligases have been classified into HECT (homologous to E6-AP C terminus) domain E3 ligases, single-subunit RING finger E3 ligases, and multisubunit RING finger E3 ligases. In the ubiquitination process, HECT E3 ligases form thioester catalytic intermediates with ubiquitin, whereas RING finger E3 ligases facilitate direct transfer of ubiquitin from E2 to substrate, probably without formation of covalent intermediates. The SCF (SKP1/cullin/F-box protein) complex belongs to the multisubunit RING finger E3 ligases, which are composed of CUL1, SKP1, RBX1, and an F-box protein. CUL1 functions as a scaffold and interacts with SKP1 and RBX1 at its N and C termini, respectively. RBX1 is a RING finger protein that recruits E2 to the E3 complex. SKP1 interacts with the F-box domain of an F-box protein, which mediates substrate recognition via another interaction domain (21).

In this study, we demonstrate that hGCMa can be ubiquitinated and subsequently degraded by the 26 S proteasome. Moreover, the human F-box protein FBW2 (hFBW2) is identified to interact with hGCMa in a phosphorylation-dependent manner and to promote ubiquitination of hGCMa. The interaction between hGCMa and hFBW2 and the critical role of hFBW2 in proteasome-mediated degradation of hGCMa are further characterized by domain mapping and RNA interference (RNAi). Our study identifies the SCF^{hFBW2} E3 complex as the key machinery that targets hGCMa to the ubiquitin-proteasome degradation system.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The GCMa expression plasmid phGCMa-FLAG, encoding full-length hGCMa plus a triple FLAG tag at its C terminus, was constructed by cloning the open reading frame of hGCMa into p3XFLAG-CMV14 (Sigma). The pHA-hGCMa expression plasmid was constructed by cloning N-terminally triple hemagglutinin (HA)-tagged full-length hGCMa into p3XFLAG-CMV14. pCS2-HA-HsSKP1 (renamed pHA-hSKP1 in this study) and pcDNA3.1/Zeo-Py2His6-CUL1 were kindly provided by Dr. R. J. Deshaies (California Institute of Technology, Pasadena, CA). The open reading frame of CUL1 was PCR-amplified from pcDNA3.1/Zeo-Py2His6-CUL1 and subcloned into p3XFLAG-CMV14 to generate phCUL1-FLAG. The cDNAs encoding the F-box proteins hFBW2, hSKP2, hFBL3A, hβTrcp, and hFBX7 were PCR-amplified from a human placental cDNA library or expressed sequence tag clones and subcloned into a cytomegalovirus expression vector containing four copies of the Myc tag at the C termini. The wild-type and mutant pGal4-hGCMa-FLAG expression plasmids were constructed by first cloning the Gal4 DNA-binding domain into p3XFLAG-CMV14 and subsequently the cDNA fragments of full-length hGCMa or its deletion mutants (see Fig. 4*A*, *lower panel*). All constructs were verified by DNA sequencing using the dideoxy chain termination method.

Cell Culture and Transfection—The 293T cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained at 37 °C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. The BeWo cell line was obtained from American Type Culture Collection and maintained at 37 °C in Kaighn's modification of Ham's F-12 medium supplemented with 15% fetal bovine serum and the same antibiotics mentioned above. To study the effects of protease inhibitors on hGCMa protein stability, 293T cells in 60-mm culture dishes were transfected with phGCMa-FLAG by calcium phosphate/DNA coprecipitation at 37 °C for 18 h and treated with 40 μM MG132, *N*-acetyl-Leu-Leu-norleucinal, or *N*-acetyl-Leu-Leu-methioninal for another 18 h. The protein level of hGCMa-FLAG was detected by Western analysis using mouse anti-FLAG monoclonal antibody (mAb) (Sigma). For reprobing, the membranes were incubated in a stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS at 50 °C for 30 min. The stripped membranes were probed with mouse anti-β-actin mAb (Sigma).

Stable BeWo lines expressing HA-hGCMa were established using a retroviral vector as described by Bender *et al.* (22). To study the stability of HA-hGCMa in BeWo cells, stable BeWo cells expressing HA-hGCMa were grown in 6-well culture plates and treated with 200 μM cycloheximide for 2 h prior to incubation with or without 40 μM MG132 for the indicated times. HA-hGCMa proteins were detected by Western analysis with rat anti-HA mAb (Roche Applied Science).

In Vivo Ubiquitination Assay—To study ubiquitination of hGCMa, 293T cells were cotransfected with pHA-hGCMa and the His-tagged ubiquitin (Ub) expression plasmid pHis-Ub for 18 h and treated with or without 40 μM MG132 for another 18 h at 37 °C. Cells were harvested for purification of His-ubiquitinated proteins by immobilized metal affinity chromatography essentially as described by Aberle *et al.* (23).

Co-immunoprecipitation—To study the interaction of hGCMa and components of the SCF complex, 293T cells were transfected with pHA-hGCMa and phCUL1-FLAG. Cells were harvested in lysis buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 6 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 20 mM NaF, 10 mM $Na_4P_2O_7$, and a protease inhibitor mixture (Sigma)) 48 h post-transfection. Aliquots were taken as input samples for Western analysis, and the remainder were subjected to immunoprecipitation with anti-HA mAb. After five extensive washings, *i.e.* shaking for 4 min at room temperature per wash with lysis buffer A, the immune complexes were analyzed by Western analysis with anti-FLAG mAb. A reciprocal experiment was performed for 293T cells transfected with pHA-hGCMa, phCUL1-FLAG, and pHA-hSKP1 by immunoprecipitation with anti-FLAG mAb and Western analysis with anti-HA mAb. To determine whether an F-box protein was interacting with hGCMa, 293T cells were cotransfected with pHA-hGCMa and phFBW2-Myc, phSKP2-Myc, phFBL3A-Myc, phβTrcp-Myc, or phFBX7-Myc. Immunoprecipitations were performed using anti-HA mAb, and the immune complexes were detected by Western analysis using mouse anti-Myc mAb (Roche Applied Science).

Metabolic Labeling Using [^{32}P]Orthophosphate and in Vitro Pull-down Assays—To study phosphorylation of hGCMa *in vivo*, 293T cells were transfected with pHA-hGCMa for 24 h and then incubated with phosphate-free medium supplemented with 4% dialyzed fetal bovine serum plus 100 μCi/ml [^{32}P]orthophosphate for 16 h. HA-hGCMa was immunoprecipitated with anti-HA mAb as described above. Dephosphorylation of HA-phospho-hGCMa was performed by treating the immune complexes with 400 units of λ-protein phosphatase (λ-PPase; New England Biolabs Inc., Beverly, MA) at 30 °C for 1 h. The reaction was analyzed by SDS-PAGE and autoradiography.

To study the effect of phosphorylation of hGCMa on the interaction of hGCMa and hFBW2, 293T cells were cotransfected with pHA-hGCMa and phFBW2-Myc or phSKP2-Myc for 48 h. Cells were harvested in lysis buffer B (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% Nonidet P-40, 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 20 mM NaF, 10 mM $Na_4P_2O_7$, 0.5 mM dithiothreitol, and a protease inhibitor mixture) or modified lysis buffer B without the phosphatase inhibitors (0.5 mM Na_3VO_4, 20 mM NaF, and 10 mM $Na_4P_2O_7$) and treated with 1200 units of λ-PPase, followed by immunoprecipitation with anti-HA mAb and Western analysis with anti-Myc mAb. For *in vitro* pull-down experiments, HA-hGCMa proteins immunopurified from the pHA-hGCMa-transfected 293T cells were treated with or without 800 units of λ-PPase and incubated with 2.5 μg of glutathione *S*-transferase (GST)-hFBW2 or GST in lysis buffer B at 4 °C for 2 h. After extensive washing, the immune complexes were analyzed by Western analysis using rabbit anti-GST polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Mapping the Interaction Domains of hGCMa and hFBW2—To map the domain of hGCMa that interacts with hFBW2, 293T cells were cotransfected with phFBW2-Myc and pGal4-hGCMa-FLAG or its dele-

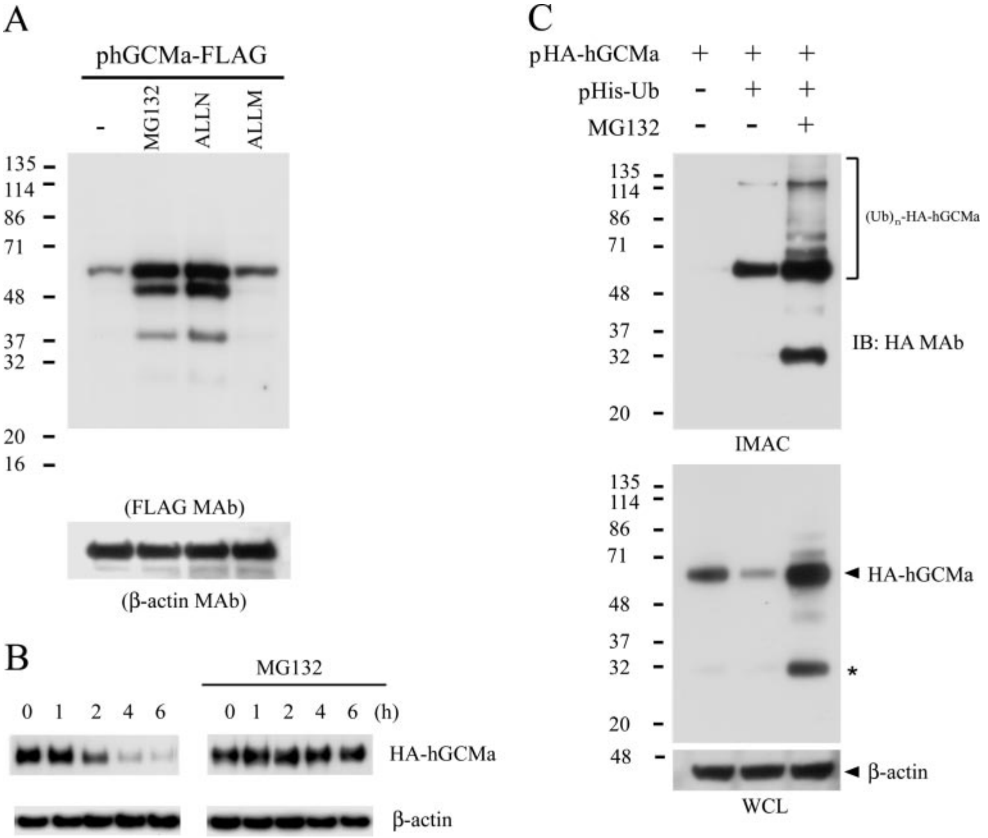

FIG. 1. **Protein degradation of hGCMa is mediated by the ubiquitin-proteasome degradation system.** *A*, the 26 S proteasome inhibitor increases the protein level of hGCMa. 293T cells were transfected with 5 μg of phGCMa-FLAG and treated with or without of the indicated protease inhibitor (40 μM). Approximately 20 μg of cell lysate were analyzed by Western analysis using anti-FLAG mAb. As a loading control, β-actin protein in the lysate was detected with anti-β-actin mAb. The small fragments in MG132- and *N*-acetyl-Leu-Leu-norleucinal (*ALLN*)-treated lanes are partially degraded hGCMa-FLAG fragments. *ALLM*, *N*-acetyl-Leu-Leu-methioninal. *B*, the 26 S proteasome inhibitor MG132 stabilizes hGCMa proteins in placental cells. Stable BeWo cells expressing HA-hGCMa were treated with 200 μM cycloheximide alone or together with 40 μM MG132 for the indicated times. Approximately 40 μg of cell lysate were analyzed by Western analysis using anti-HA mAb. The protein level of β-actin is shown as a loading control. *C*, *in vivo* ubiquitination of hGCMa. 293T cells were transfected with 5 μg of pHA-hGCMa alone or together with 1 μg of pHis-Ub and treated with or without 40 μM MG132. Ubiquitinated HA-hGCMa proteins in the cell lysate were purified by immobilized metal affinity chromatography (*IMAC*) and subsequently detected by Western analysis using anti-HA mAb. The protein levels of HA-hGCMa and β-actin in the whole cell lysate (*WCL*) were detected by Western analysis. The *asterisk* indicates a partially degraded HA-hGCMa fragment. *IB*, immunoblot.

tion mutant plasmids. Co-immunoprecipitation was performed using anti-Myc mAb, and the immune complexes were analyzed by Western analysis using anti-FLAG mAb. To map the domain of hFBW2 that interacts with hGCMa, full-length hFBW2 and its deletion mutants were cloned into the expression vector pGEX6P-1 (Amersham Biosciences) (see Fig. 4*B*, *lower panel*). GST-hFBW2 fusion proteins were expressed in *Escherichia coli* BL21(DE3) cells and purified on glutathione 4B-Sepharose (Amersham Biosciences) according to the manufacturer's instructions. HA-hGCMa-containing cell lysate was prepared by harvesting pHA-hGCMa-transfected 293T cells in lysis buffer C (20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.05% Tween 20, 5% glycerol, 1 mM Na_3VO_4, 5 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). GST pull-down experiments were performed by incubating 180 μg of cell lysate and 2.5 μg of GST or the indicated GST-hFBW2 fusion protein in lysis buffer C containing glutathione beads at 4 °C for 18 h. After extensive washing, the beads were analyzed by Western analysis using anti-HA mAb.

Gene Silencing by RNAi—For generation of small interfering RNA (siRNA) for hFBW2, the gene-specific oligonucleotides 910 (5′-AGATGGACTTCTCTGTACAGG-3′) and 1112 (5′-GACATTGTCTGTCTCTGAGGA-3′), followed by a non-complementary spacer (5′-GAGTCGTCG-3′) and the reverse complements of the oligonucleotide 910 and 1112 sequences, were synthesized and cloned into the pSuppressorRetro plasmid (Imgenex Corp., San Diego, CA). The resultant psiRNA910 and psiRNA1112 expression plasmids were characterized for silencing exogenous and endogenous hFBW2 expression (see Fig. 5, *A* and *B*). An unrelated siRNA expression plasmid (psiRNANC; Imgenex Corp.) harboring a sequence (5′-TCAGTCACGTTAATGGTCGTT-3′) showing no significant homology to human gene sequences was used as a negative control.

To study the effect of gene silencing of hFBW2 on the ubiquitination of hGCMa, 293T cells were transfected with different combinations of phGCMa-FLAG, pHA-Ub (kindly provided by Dr. A. Moustakas, Ludwig Institute for Cancer Research, Uppsala, Sweden), psiRNA910, and psiRNA1112 as indicated in the figure legend to Fig. 5*C* and treated with MG132. Analysis of ubiquitinated hGCMa was performed by immunoprecipitation using anti-HA mAb and by Western analysis using anti-FLAG mAb. In addition, a reciprocal experiment was performed by immunoprecipitation using anti-FLAG mAb and by Western analysis using anti-HA mAb. To study the effect of gene silencing of hFBW2 on the protein stability of hGCMa, 293T cells were transfected with pHA-hGCMa plus psiRNANC or psiRNA910. 36 h post-transfection, cells were pulse-labeled with 50 μCi/ml [^{35}S]methionine for 2 h and chased with unlabeled methionine for the indicated times. Cells were harvested for immunoprecipitation with anti-HA mAb and analyzed by SDS-PAGE and fluorography.

RESULTS

The Proteasome Is Involved in the Degradation of hGCMa—To test whether the hGCMa protein is degraded by the 26 S proteasome degradation system, 293T cells were transfected with phGCMa-FLAG and treated with or without protease inhibitors, including *N*-acetyl-Leu-Leu-methioninal, *N*-acetyl-Leu-Leu-norleucinal, and MG132. As shown in Fig. 1*A*, *N*-acetyl-Leu-Leu-methioninal, an inhibitor of cathepsins and calpains, did not affect the protein level of hGCMa-FLAG compared with the untreated control. However, *N*-acetyl-Leu-Leu-norleucinal, an inhibitor of calpains and the 26 S protea-

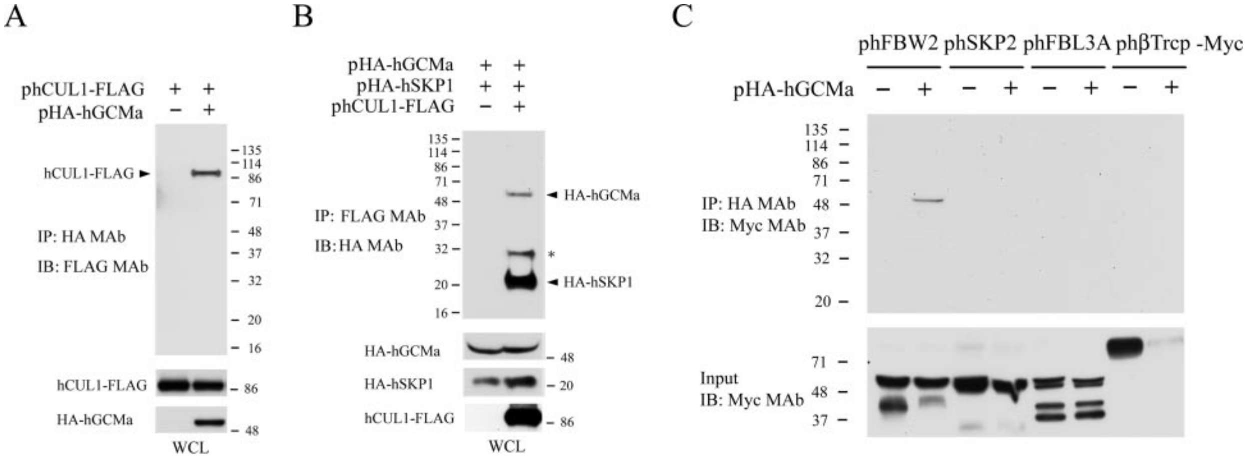

FIG. 2. **hGCMa associates with components of the SCF complex.** *A* and *B*, hGCMa associates with hCUL1 and hSKP1 *in vivo*. 293T cells were transfected with 5 μg of pHA-hGCMa alone or together with 1 μg of phCUL1-FLAG (*A*) or the indicated combinations of 5 μg of pHA-hGCMa, 1 μg of pHA-hSKP1, and 1 μg of phCUL1-FLAG (*B*). Co-immunoprecipitation assays were performed as described under "Experimental Procedures." The *asterisk* indicates a partially degraded HA-hGCMa fragment. The protein levels of HA-hGCMa, hCUL1-FLAG, and HA-SKP1 in the whole cell lysate (*WCL*) were detected by Western analysis. *C*, identification of a human F-box protein recognizing hGCMa. 293T cells were transfected with 3.5 μg of the indicated F-box protein expression plasmid alone or together with 3.5 μg of pHA-hGCMa. Co-immunoprecipitation assays were performed as described under "Experimental Procedures." The input protein level of F-box proteins was detected by Western analysis using anti-Myc mAb. *IP*, immunoprecipitation; *IB*, immunoblot.

some, significantly increased the protein level of hGCMa-FLAG. Similarly, MG132, an inhibitor of the 26 S proteasome, also increased the protein level of hGCMa-FLAG. These results suggest that the protein level of hGCMa can be regulated by the 26 S proteasome degradation system *in vivo*.

Because GCMa is primarily expressed in placenta, we next tested whether hGCMa is also degraded by the 26 S proteasome degradation system in placental cells. To this end, the BeWo human placental cell line was used to establish stable lines expressing HA-hGCMa via a retroviral expression system. Stable HA-hGCMa-expressing BeWo cells were treated with cycloheximide and incubated for different periods of time in the presence or absence of MG132. As shown in Fig. 1*B*, HA-GCMa maintained a half-life of between 1 and 2 h in BeWo cells in the absence of MG132. However, the HA-GCMa protein level remained unchanged for 6 h in the presence of MG132 (Fig. 1*B*). These results suggest that the GCMa protein is degraded by the 26 S proteasome degradation system in placental cells.

Because the 26 S proteasome mediates the degradation of ubiquitinated proteins, we tested whether hGCMa is ubiquitinated *in vivo*. To this end, 293T cells were transfected with pHA-hGCMa and pHis-Ub and treated with or without MG132. Ubiquitinated proteins in the cell lysate from transfected 293T cells were then purified and enriched by immobilized metal affinity chromatography. The level of ubiquitinated HA-hGCMa in the purified ubiquitinated proteins was further detected by Western analysis. As shown in Fig. 1*C* (*lower panel*), the protein level of HA-hGCMa decreased when His-Ub was coexpressed, which is very likely due to a higher level of His-Ub promoting protein degradation. In contrast, the protein level of HA-hGCMa increased in the presence of MG132 even though His-Ub was coexpressed (Fig. 1*C*, *lower panel*). In the analysis of GCMa ubiquitination, no signal for His-ubiquitinated HA-GCMa was detected in cells transfected with pHA-hGCMa alone, as expected (Fig. 1*C*, *upper panel*). However, signals for His-ubiquitinated HA-GCMa were detected in the HA-hGCMa and His-Ub coexpression group and were further enhanced in the presence of MG132 (Fig. 1*C*, *upper panel*). These results suggest that hGCMa can be ubiquitinated *in vivo*. Likewise, *Drosophila* GCM1 and mouse GCMb were also ubiquitinated when His-Ub was coexpressed with them in the presence of MG-132 (data not shown). Taken together, these results suggest that GCM proteins can be ubiquitinated and degraded by the 26 S proteasome.

Characterization of the F-box Protein Interacting with hGCMa—Because the SCF E3 complex is involved in the degradation of many transcription factors, we now tested whether an SCF E3 complex is required for the degradation of hGCMa. We first tested whether hGCMa interacts with components of the SCF complex by transfecting 293T cells with pHA-hGCMa and phCUL1-FLAG in co-immunoprecipitation assays. As shown in Fig. 2*A*, the hCUL1-FLAG protein was detected in the precipitated HA-hGCMa complex. Furthermore, a reciprocal co-immunoprecipitation experiment was performed using 293T cells transfected with pHA-hGCMa, phCUL1-FLAG, and pHA-hSKP1. An interaction among HA-GCMa, hCUL1-FLAG, and HA-SKP1 was also observed (Fig. 2*B*). These results suggest that hGCMa associates with the SCF E3 complex *in vivo*.

Because the substrate specificity for an SCF E3 complex depends on its associated F-box protein, we next identified the F-box protein that recognizes hGCMa. We searched the literature for several human F-box proteins known to be expressed in placenta, including hSKP2, hFBW2, hFBL3A, hβTrcp, and hFBX7, and tested them for interaction with hGCMa in co-immunoprecipitation assays. We detected a specific interaction between hGCMa and hFBW2, but not hSKP2, hFBL3A, or hβTrcp (Fig. 2*C*) or hFBX7 (data not shown). These results suggest that hFBW2 is the F-box protein that specifically recognizes hGCMa.

Phosphorylation-dependent Interaction between hGCMa and hFBW2—We further tested whether phosphorylation of hGCMa is required for interaction with hFBW2 because several well characterized F-box proteins such as βTrcp, SKP2, andFBW7recognizetheircognatesubstratesinaphosphorylation-dependent manner (23–32). We first investigated whether hGCMa is a phosphoprotein by *in vivo* metabolic labeling of hGCMa with [^{32}P]orthophosphate in 293T cells transfected with pHA-hGCMa. As shown in Fig. 3*A*, the HA-hGCMa protein was phosphorylated in 293T cells, and its phosphate moieties could be cleaved off by λ-PPase. Therefore, hGCMa is a phosphoprotein *in vivo*. We then tested whether phosphorylation of hGCMa is required for interaction with hFBW2. To this end, 293T cells were first transfected with pHA-hGCMa and phFBW2-Myc or phSKP2-Myc. Then, the cell lysate from transfected 293 cells was collected in the presence or absence of

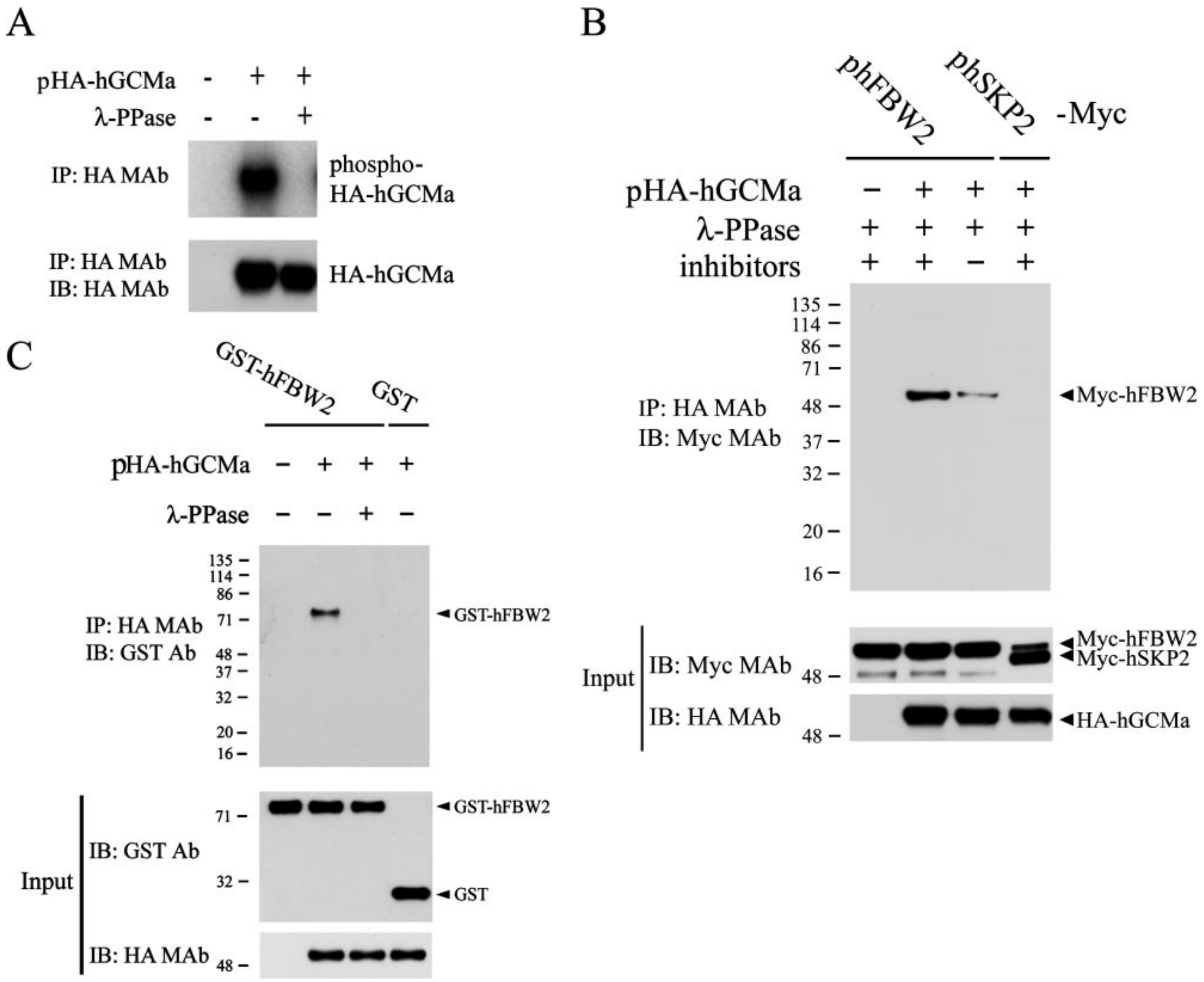

FIG. 3. **Interaction between hFBW2 and hGCMa is phosphorylation-dependent.** *A*, hGCMa is a phosphoprotein *in vivo*. 293T cells were transfected with 5 μg of pHA-hGCMa and pulse-labeled with 100 μCi/ml of [^{32}P]orthophosphate for 16 h. Phosphorylated HA-hGCMa proteins were immunoprecipitated (*IP*) with anti-HA mAb. The immune complex was then treated with or without 400 units of λ-PPase and analyzed by SDS-PAGE and autoradiography. *IB*, immunoblot. *B* and *C*, phosphorylation-dependent interaction between hGCMa and hFBW2. In *B*, 293T cells were transfected with 3.5 μg of pHA-hGCMa alone or together with 3.5 μg of the indicated F-box protein expression plasmid. After harvesting the cells in lysis buffer B with or without phosphatase inhibitors, the cell lysate was treated with 1200 units of λ-PPase, followed by co-immunoprecipitation analysis as described under "Experimental Procedures." In *C*, 293T cells were transfected with 3.5 μg of pHA-hGCMa, and the HA-hGCMa proteins were immunopurified with anti-HA mAb. The immune complex was treated with or without 800 units of λ-PPase, followed by incubation with 2.5 μg of GST or GST-hFBW2 proteins for pull-down assays as described under "Experimental Procedures."

phosphatase inhibitors and further challenged with λ-PPase, followed by co-immunoprecipitation assays. As shown in Fig. 3*B*, a specific interaction between hGCMa and hFBW2 (but not hSKP2) was observed in the presence of phosphatase inhibitors. Interestingly, this interaction was significantly diminished when the lysate was collected in the absence of phosphatase inhibitors (Fig. 3*B*), suggesting that phosphorylation of hGCMa is required for interaction with hFBW2. Additionally, pull-down experiments were performed to further confirm the phosphorylation-dependent interaction between hGCMa and hFBW2. HA-hGCMa was first immunopurified from pHA-hGCMa-transfected 293T cells and treated with or without λ-PPase, followed by incubation with GST-hFBW2 or GST. Again, a specific interaction was observed between HA-hGCMa and GST-hFBW2 (Fig. 3*C*). However, this interaction was abolished when the immunopurified HA-hGCMa protein was pretreated with λ-PPase (Fig. 3*C*). No interaction was seen between GST and HA-hGCMa either pretreated or not with λ-PPase (Fig. 3*C*). Taken together, these results strongly suggest that hFBW2 preferentially recognizes the phosphorylated form of the hGCMa protein.

Identification of the Interaction Domains of hGCMa and FBW2—We then mapped the interaction domains of hGCMa and hFBW2. To map the hFBW2-interacting domain of hGCMa, expression plasmids harboring the Gal4 DNA-binding domain fused with full-length hGCMa-FLAG or its deletion mutants (Fig. 4*A*, *lower panel*) were cotransfected with phFBW2-Myc into 293T cells. Interactions between hFBW2-Myc and Gal4-hGCMa-FLAG or its deletion mutants were analyzed by co-immunoprecipitation assays. As shown in Fig. 4*A*, an interaction was detected between hFBW2 and full-length hGCMa as well as the C-terminal deletion mutants containing amino acids 1–300 and 1–167. However, the N-terminal deletion mutants containing amino acids 167–436 and 300–436 did not interact with hFBW2. Therefore, the hFBW2-interacting domain of hGCMa was identified as region 1–167, which also harbors the GCM motif.

To map the hGCMa-interacting domain of hFBW2, GST fusion proteins of wild-type hFBW2 and hFBW2 deletion mutants (Fig. 4*B*, *lower panel*) were prepared from *E. coli* and incubated with 293T cell lysate containing HA-hGCMa for GST pull-down assays. The hFBW2 polypeptide consists of an F-box

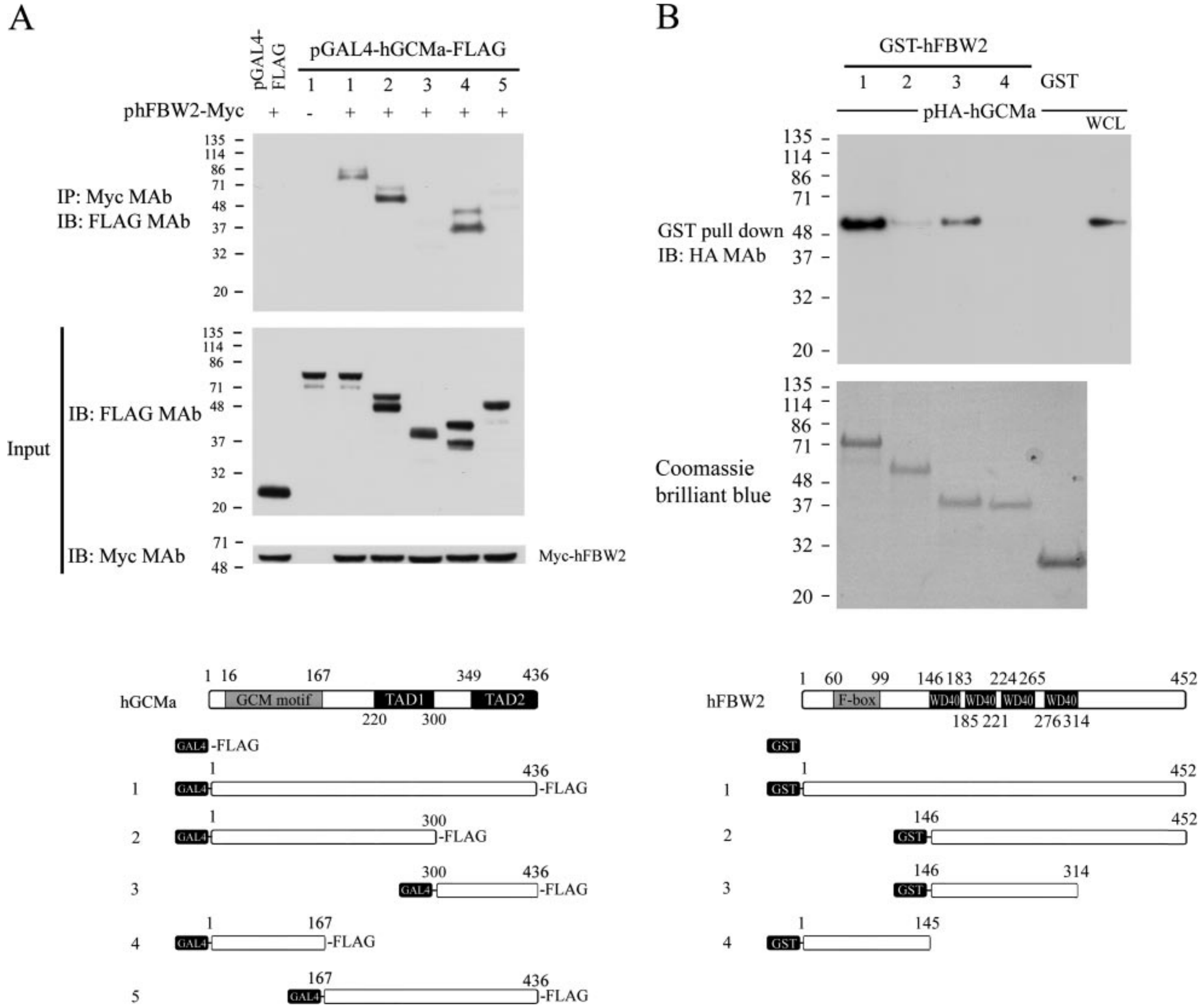

FIG. 4. **Mapping of the interaction domains of hGCMa and hFBW2.** *A*, the GCM motif of hGCMa is the interaction domain for hFBW2. 293T cells were transfected with 3.5 μg of phFBW2-Myc and 3.5 μg of the indicated expression plasmids for wild-type and mutant Gal4-hGCMa-FLAG fusion proteins. Co-immunoprecipitation assays were performed as described under "Experimental Procedures." The results from Western analyses of hFBW2-Myc and wild-type and mutant Gal4-hGCMa-FLAG fusion proteins in one-twentieth of input cell lysate for co-immunoprecipitation assays are presented. A schematic representation of the pGal4-hGCMa-FLAG expression plasmids is shown below. *TAD*, transactivation domain; *IP*, immunoprecipitation; *IB*, immunoblot. *B*, the WD40 repetitive region of hFBW2 is the interaction domain for hGCMa. 293T cells were transfected with 5 μg of pHA-hGCMa. 48 h post-transfection, cells were harvested for GST or GST-hFBW2 pull-down assays as described under "Experimental Procedures." The results from Western analysis of one-twentieth of input whole cell lysate (*WCL*) and Coomassie Brilliant Blue R-250 staining of wild-type and mutant GST-hFBW2 fusion proteins for pull-down assays are presented. A schematic representation of the GST-hFBW2 fusion protein and its deletion mutants is shown below.

and four WD40 repeats. The F-box motif interacts with SKP1 of the SCF complex, whereas the WD40 repeats are believed to mediate interaction with protein substrates. As shown in Fig. 4*B*, the N-terminal region (amino acids 1–145) of the hFBW2 polypeptide containing the F-box did not interact with HA-hGCMa. However, the hFBW2 N-terminal deletion mutants containing amino acids 146–452 and 146–314 interacted with HA-hGCMa (Fig. 4*B*). Interestingly, the latter had the highest binding activity for HA-hGCMa, suggesting that amino acids 315–452 C-terminal to the WD40 repeats may regulate substrate recognition. These results suggest that the hGCMa-interacting domain of hFBW2 is region 146–314, which harbors the WD40 repeats.

FBW2 Mediates the Ubiquitination of hGCMa—Because we found that hGCMa interacts with hFBW2, it seemed very likely that hFBW2 of the $\text{SCF}^{\text{hFBW2}}$ E3 complex is involved in the ubiquitination of hGCMa for proteasome degradation. To test this hypothesis, we used RNAi to knock down the protein level of hFBW2 *in vivo* and tested whether ubiquitination of hGCMa was inhibited when the protein level of hFBW2 was reduced. We first established RNAi for hFBW2. Two siRNA expression plasmids (psiRNA910 and psiRNA1112) derived from the hFBW2 nucleic acid sequence were tested for their ability to silence expression of exogenous hFBW2-Myc and endogenous hFBW2 in 293T cells. As shown in Fig. 5*A*, psiRNA910 (but not psiRNA1112 or psiRNANC, an unrelated negative control) was highly efficient in reducing the protein level of exogenous hFBW2-Myc. In addition, psiRNA910, psiRNA1112, and psiRNANC were tested for their ability to silence the endogenous hFBW2 protein in 293T cells pulse-labeled with [^{35}S]methionine. As shown in Fig. 5*B*, the protein level of endogenous hFBW2 was significantly reduced by psiRNA910, but not by psiRNA1112 or psiRNANC.

We then tested whether ubiquitination of hGCMa is inhibited when expression of the h*FBW2* gene is silenced. 293T cells were transfected with phGCMa-FLAG alone or together with pHA-Ub plus psiRNA910 or psiRNA1112, treated with MG132, and analyzed by co-immunoprecipitation assays. As shown in

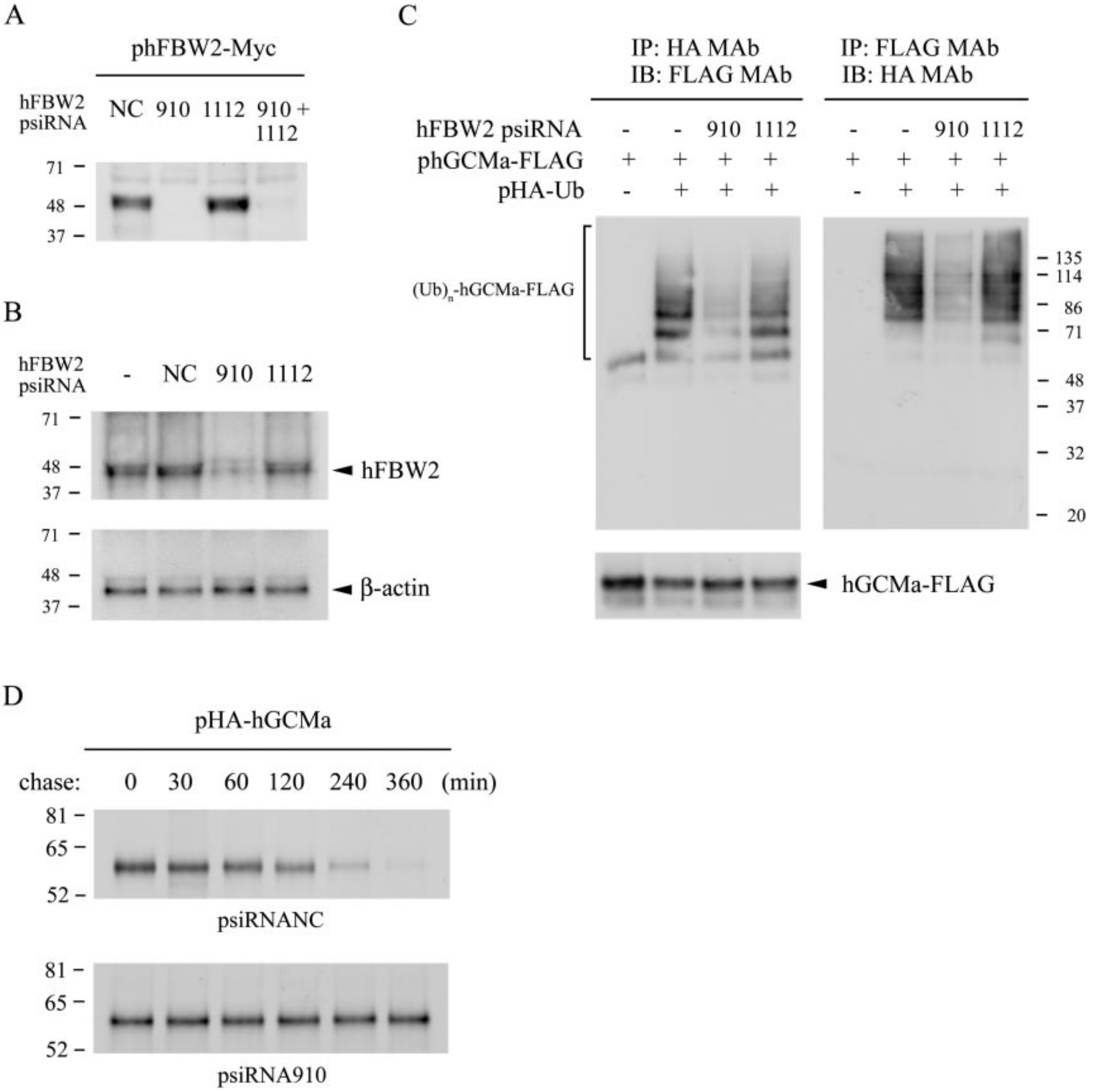

FIG. 5. **hFBW2 promotes the ubiquitination of hGCMa *in vivo*.** *A* and *B*, RNAi of hFBW2. 293T cells were cotransfected with 2 μg of phFBW2-Myc and 4 μg of the indicated psiRNA expression plasmid. 48 h post-transfection, cells were harvested for Western analysis of hFBW2-Myc using anti-Myc mAb (*A*). 293T cells were transfected with 4 μg of the indicated siRNA expression plasmid for 18 h and then pulse-labeled with 20 μCi/ml [^{35}S]methionine for 16 h, followed by immunoprecipitation using guinea pig anti-hFBW2 antibody or anti-β-actin mAb. The immune complexes were analyzed by SDS-PAGE and fluorography (*B*). *C*, effect of hFBW2 on the ubiquitination of hGCMa. 293T cells were transfected with 3.5 μg of phGCMa-FLAG alone or together with 3 μg of pHA-Ub plus 3.5 μg of psiRNA910 (*910*) or psiRNA1112 (*1112*). 18 h post-transfection, cells were treated with 40 μM MG132 for another 18 h and then analyzed by co-immunoprecipitation assays as described under "Experimental Procedures." Ubiquitinated hGCMa-FLAG proteins are *bracketed*. The protein level of hGCMa-FLAG in whole cell lysates was analyzed by Western analysis. *IP*, immunoprecipitation; *IB*, immunoblot. *D*, the protein stability of hGCMa is increased in the presence of RNAi silencing of hFBW2. 293T cells were transfected with 3.5 μg of pHA-hGCMa plus 3.5 μg of psiRNANC (*NC*) or psiRNA910. 36 h post-transfection, cells were analyzed in pulse-chase experiments as described under "Experimental Procedures."

Fig. 5*C* (*right panel*), after immunopurification of hGCMa-FLAG, the level of ubiquitinated hGCMa-FLAG was significantly reduced by the addition of psiRNA910, but not psiRNA1112. A reciprocal co-immunoprecipitation experiment was performed to detect the level of ubiquitinated hGCMa-FLAG in the immunopurified ubiquitinated proteins. Similarly, the level of ubiquitinated hGCMa-FLAG was significantly reduced by the addition of psiRNA910, but not psiRNA1112 (Fig. 5*C*, *left panel*). Taken together, these results suggest that hFBW2 is involved in the ubiquitination of hGCMa *in vivo*.

We further examined whether the protein stability of hGCMa is increased when expression of the h*FBW2* gene is effectively silenced. Pulse-chase experiments with hGCMa were performed in 293T cells transfected with pHA-hGCMa plus psiRNANC or psiRNA910. The half-life of HA-GCMa in 293T cells cotransfected with pHA-hGCMa and psiRNANC was between ~1 and 2 h, whereas it was significantly extended as long as 6 h in 293T cells cotransfected with pHA-hGCMa and psiRNA910 (Fig. 5*D*). These results suggest that hGCMa is significantly stabilized with effective knockdown of h*FBW2* gene expression.

DISCUSSION

GCMa is primarily expressed in placenta and plays a key role in mediating trophoblast cell differentiation. In this study, we first provided evidence that hGCMa can be ubiquitinated and degraded by the 26 S proteasome degradation system by demonstrating that MG132, a 26 S proteasome inhibitor, can increase the levels of the hGCMa protein and its polyubiquitinated form *in vivo*. Subsequently, we demonstrated that an SCF complex is involved in the regulation of hGCMa turnover because hGCMa associates with two key components of the complex (CUL1 and SKP1) *in vivo*. We further screened several human F-box proteins expressed in placenta for interactions with hGCMa and identified hFBW2 as the F-box protein for hGCMa *in vivo* and *in vitro*. To our knowledge, hGCMa is the first identified substrate protein of hFBW2.

Currently, >70 proteins harboring a conserved 40-amino acid domain termed the F-box have been identified in the human genome data base (33–35). Based on the amino acid sequence, F-box proteins are further classified into three classes: FBW proteins contain WD40 repeats; FBL proteins contain leucine-rich repeats; and FBX proteins lack known protein-interacting domains (21, 33–35). hFBW2, also known as MD6, contains four WD40 repeats and was first reported by Bai *et al.* (36) after they retrieved F-box-containing sequences from data bases. Studies have indicated that phosphorylation of substrate proteins is required for their interaction with several F-box proteins (23–32). For example, βTrcp recognizes the phosphorylated $DSG(X)_{2+n}S$ motif within its substrates, including IκB and β-catenin (23–26). In this study, we demonstrated that hGCMa is a phosphoprotein *in vivo* and that phosphorylation of hGCMa is required for interaction with hFBW2 because dephosphorylation of hGCMa by λ-PPase completely abolished this interaction. More recently, phosphorylation of c-Myc at Thr^{58} by glycogen synthase kinase-3 was shown to be required for binding of FBW7 to c-Myc as well as FBW7-mediated c-Myc ubiquitination and degradation (30, 31). Currently, it is not clear which signaling pathways regulate phosphorylation of hGCMa, nor is it clear how this modification regulates the recognition of hGCMa by hFBW2. Further investigation is required to answer these intriguing questions.

It is known that ubiquitination of a substrate protein may be mediated by more than one F-box protein. For example, c-Myc turnover is regulated by SKP2 and FBW7 through two different regions of the c-Myc polypeptide (29–32). To a lesser degree, we could still detect ubiquitinated hGCMa proteins *in vivo* in the presence of the functional hFBW2 siRNA expression plasmid psiRNA910 (Fig. 5*C*). A possible explanation for this observation is that the efficiency of the hFBW2 siRNA is not high enough to completely silence the h*FBW2* gene expression. Future study using FBW2 knockout cells may help to verify this possibility. However, the possibility that extra F-box proteins other than hFBW2 are involved in the ubiquitination of hGCMa cannot be ruled out.

Because the ubiquitin-proteasome degradation system plays an important role in many cellular processes, aberrant regulation of F-box proteins may cause physiological abnormalities. For example, SKP2 mediates proteolysis of the cyclin-dependent kinase inhibitors p27 and p21 (37, 38). However, amplification and overexpression of the *SKP2* gene have been found in a wide spectrum of tumors (39). Accordingly, *SKP2* has been classified as an oncogene with growth-promoting abilities; and clinically, elevated expression of the *SKP2* gene has been an indicator of poor prognoses for cancer patients (39). In contrast, FBW7 mediates proteolysis of cyclin E, which is overexpressed in breast cancer (28). The observation that expression of the *FBW7* gene is decreased in breast tumor lines suggests that FBW7 is a potential tumor suppressor protein (28). Given that hGCMa regulates syncytin-mediated trophoblastic fusion, it is feasible to speculate that abnormal expression of *FBW2* may impede placental development. Specifically, we have recently demonstrated that decreased levels of hGCMa proteins are found in pre-eclamptic placentas (40). Although this abnormality can be attributed to decreased expression of the h*GCMa* gene, aberrant degradation of hGCMa could also be a possible cause of decreased levels of hGCMa proteins in pre-eclamptic placentas and warrants further investigation. Overall, this study has demonstrated that the SCF^{hFBW2} E3 complex is the key machinery that targets hGCMa to the ubiquitin-proteasome degradation system and has revealed a potential post-translational control of GCMa activity during placental development in terms of protein turnover.

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