Small Ubiquitin-like Modifier Modification Regulates the DNA Binding Activity of Glial Cell Missing Drosophila Homolog a

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Glia missing Drosophila homolog a (GCMa) is an essential transcription factor for placental development, which controls the differentiation of the syncytiotrophoblast layer. Although the activity of GCMa can be post-translationally regulated by protein phosphorylation, ubiquitination, and acetylation, it is unknown whether GCMa activity can be regulated by sumoylation. In this report, we investigated the role of sumoylation in the regulation of GCMa activity. We demonstrated that Ubc9, the E2 component of the sumoylation machinery, specifically interacts with the N-terminal domain of GCMa and promotes GCMa sumoylation on lysine 156. Moreover, GCMa-mediated transcriptional activation was repressed by sumoylation but was enhanced in the presence of the SUMO-specific protease, SENP1. The repressive effect of sumoylation on GCMa transcriptional activity was attributed to decreased DNA binding activity of GCMa. Furthermore, structural analysis revealed a steric clash between the SUMO1 moiety of sumoylated GCMa and the DNA-binding surfaces of GCMa, which may destabilize the interaction between GCMa and its cognate DNA sequence. Our study demonstrates that GCMa is a new sumoylation substrate and its activity is down-regulated by sumoylation.

The multinucleated syncytiotrophoblasts covering placental villi are essential for maternal-fetal exchange of gas and nutrients. The placental glial cell missing Drosophila homolog a (GCMa)2 transcription factor regulates expression of syncytin, which is a fusogenic protein mediating cell-cell fusion of monocytic cytotrophoblasts to form syncytiotrophoblasts (1).

Because syncytiotrophoblasts undergo apoptosis, to maintain the structural and functional integrity of placental villi, GCMa very likely plays a pivotal role in regulation of syncytiotrophoblast formation. Indeed, GCMa activity is regulated by post-translational modifications, which may in turn fine tune the differentiation of syncytiotrophoblast. Recently, we have demonstrated that CREB-binding protein directly interacts with and acetylates GCMa in the activated cAMP/protein kinase A signaling pathway (2). Acetylation of GCMa prolongs its protein stability with a concomitant increase in transcriptional activity, which may provide an explanation of why the cAMP stimulant, forskolin, enhances placental cell fusion. Because acetylation is a reversible modification, several histone deacetylases (HDAC1, -3, -4, and -5) have been identified to mediate deacetylation of GCMa (3). On the other hand, in vitro labeling experiments have also revealed that GCMa is a phosphoprotein. Being phosphorylated, GCMa can be targeted by the F-box protein, FBW2, and subjected to protein degradation by the ubiquitin-proteasome degradation system (4). Therefore, regulation of GCMa activity is achieved at multiple levels by phosphorylation, acetylation, deacetylation, and ubiquitination.

Sumoylation is a protein modification involving a covalent conjugation of a polypeptide termed the small ubiquitin-like modifier (SUMO) to lysine residues of target proteins (5, 6). SUMO proteins are structurally related to ubiquitin and are expressed as precursors that undergo proteolytic cleavage to make the C-terminal glycine-glycine motif available for conjugation. At least three mammalian SUMO proteins, SUMO1, -2, and -3 have been identified. SUMO2 and -3 share greater than 90% identity in amino acid sequence, whereas both are about 50% identical to SUMO1. Similar to ubiquitination, sumoylation is catalyzed by a set of enzymes, including E1-activating enzyme (Aos1/Uba2 or SAE1/SAE2), E2-conjugating enzyme (Ubc9), and E3 ligases. However, unlike ubiquitination, a consensus sequence for SUMO modification has been identified as PKKXE, where Ψ is a large hydrophobic amino acid and K is the site of SUMO conjugation (5, 6). Moreover, recombinant E1, Ubc9, and SUMO are sufficient for ATP-dependent SUMO modification of substrates in vitro. Consistent with this observation, structural studies have shown direct recognition of the SUMO consensus motif by the Ubc9 active site (7). Three types of SUMO E3 ligases have been identified, including RanBP2, the PIAS proteins, and Pdc2. They exhibit different subcellular localization patterns and may be important in regulating sub-

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1-4.
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strate recognition and enhancing sumoylation of substrates in vivo. Interestingly, PIAS proteins can also function as transcriptional coregulators. With no sumoylation involved, PIAS1 has recently been shown to interact with and localize the Mxs1 homeoprotein to the nuclear periphery, where Mxs1 can engage with and repress the expression of its target genes (8).

A wide spectrum of functional consequences of SUMO modification of protein has been reported, including modulation of transcriptional activity, mediation of nuclear import, recruitment of transcriptional regulators in nuclear domains, protection from ubiquitination, and regulation of mitosis (6). In addition, conjugation of SUMO can provide new interaction surfaces to protein and facilitate proteolytic processing, respectively. For instance, sumoylation of p300 and Elk1 facilitates the recruitment of HDAC6 and HDAC2, respectively (9, 10). Sumoylation in the N-terminal inhibitory domain of Sp1 prevents proteolytic cleavage of this domain and hence represses Sp1 transcriptional activity (11). On the other hand, phosphorylation-dependent sumoylation motif of ΨKXEXXP, composed of a SUMO consensus site and an adjacent proline-directed phosphorylation site, has been identified in several transcription factors, including GATA-1 and MEF2 (12). It is foreseeable that new SUMO substrates will be identified based on the phosphorylation-dependent sumoylation motif.

Although protein phosphorylation, acetylation, deacetylation, and ubiquitination regulate GCMa activity, it remains unknown whether GCMa activity can be regulated by sumoylation. In this study, we investigated the role of sumoylation in the regulation of GCMa activity. We demonstrated that the Ubc9 protein specifically interacts with GCMa and promotes GCMa sumoylation. Furthermore, the primary SUMO acceptor site was identified as lysine 156 in the N-terminal domain of GCMa, which is also the interaction domain for Ubc9. Sumoylation of GCMa repressed the transcriptional activity of GCMa. This is most likely due to a decrease in the DNA binding activity of GCMa, since protein modeling indicated that the SUMO1 domain can impose steric hindrance on the DNA-binding surfaces of GCMa.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The expression plasmids pHAGCMa, pGCMa-FLAG, pGal4-FLAG, and pGal4-GCMa-FLAG and its deletion mutants have been described previously (2). The K149R, K156R, K160R, and E158A mutants of pGal4-GCMa-FLAG (1–300) were constructed by site-directed mutagenesis at lysine 149, lysine 156, lysine 160, and glutamic acid 158, respectively. The K3R mutant harbors a combined mutation at lysine 149, lysine 156, and lysine 160. pHA-GCMa-SUMO1 and pSUMO1-GCMa-FLAG were constructed by inserting a SUMO1 cDNA fragment in frame to the 3′-end of the HA-GCMa and to the 5′-end of the GCMa-FLAG cDNA fragment, respectively. To prevent desumoylation of the chimeric SUMO1-GCMa-FLAG protein, the codons for the last two glycine residues in the SUMO1 cDNA were changed to encode two alanine residues. pUbc9-FLAG and -Myc, pPIAS2β-Myc, and pS EPN1-FLAG were constructed by placing the respective open reading frame cDNA tagged at the C-terminal with three copies of FLAG or four copies of Myc under the control of cytomegalovirus early promoter/enhancer. pUbSac-FLAG-dn encodes a dominant-negative Ubc9 harboring a Cys39 → Ser mutation in its active site. pPIAS2β-Myc/C-S encodes a null mutant PIAS2β harboring a Cys362 → Ser mutation. pEGFP-SUMO1 was constructed by cloning the wild type SUMO1 cDNA fragment (amino acids 1–97) into pEGFP-C1 (Clontech). pEGFP-SUMO1-AAA is similar to pEGFP-SUMO1 except that the last two glycine residues were changed into two alanine residues. A syncytin promoter fragment derived from the 083M05 BAC clone was inserted into pE1bLUC to generate p(27950/28314)LUC. The proximal GCMa-binding site (pGBS, 28026–28033) in p(27950/28314)LUC was deleted to generate p(27950/28314)LUC-ΔGBS. The reporter construct, p(pGBS)E1bLUC, has been described previously (2). All constructs were verified by DNA sequencing using the dideoxy chain termination method.

Cell Culture, Transfection, and Reporter Gene Assay—293T and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37 °C in HEPES-buffered Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). JAR cells and the BeWo-derived stable line expressing HA-GCMa were maintained at 37 °C in F-12K medium supplemented with 15% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 units/ml). For transient expression experiments, cells were transfected with the amounts of plasmids described in the figure legends using calcium phosphate–DNA coprecipitation, the TransIT LT1 reagent (Mirus, Madison, WI), or the Lipofectamine 2000 reagent (Invitrogen). In addition, adjusted amounts of the empty expression vector were added to maintain a constant amount of total DNA in each transfection assay. For luciferase reporter assays, cells were harvested in the reporter lysis buffer (Promega, Madison, WI) 48 h post-transfection and analyzed as previously described (2).

Co-Immunoprecipitation, GST Pull-Down Assay, and Interaction Domain Mapping—To study the interaction between GCMa, Ubc9, and PIAS, 293T cells were cotransfected with the indicated amount of pHA-GCMa, pUbc9-FLAG, and pPIAS2β-Myc as described in the legend to Fig. 1A. 48 h post-transfection, cells were harvested for immunoprecipitation and immunoblotting as previously described (2). The glutathione S-transferase (GST) fusion protein expression vector pGEX6P-1 (Amersham Biosciences) was used for preparation of the GST-Ubc9 fusion protein in Escherichia coli strain BL21(DE3). Recombinant GCMa-FLAG protein was prepared by the baculovirus–insect cell expression system as previously described (1). To study the physical interaction between GCMa and Ubc9, 0.2 μg of GCMa-FLAG and 2.5 μg of GST or GST-Ubc9 were used for GST pull-down experiments as previously described (3). To map the interaction domain of GCMa for Ubc9, 293T cells were transfected with pUbc9-Myc and pGal4-GCMa-FLAG or its deletion mutants, followed by co-immunoprecipitation analysis.

In Vivo and In Vitro Sumoylation Assays—To study GCMa sumoylation in vivo, 293T cells were transfected with different combinations of pHA-GCMa, pEGFP-SUMO1, pEGFP-
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SUMO1-AA, pUb9-FLAG, pUb9-FLAG-dn, pPIAS2β-Myc, and pPIAS2β-Myc-C/S as described in the legend to Fig. 2A. Detection of sumoylated GCMa was performed by immunoprecipitation with HA mAb and then immunoblotting with HA or EGFP mAb. Sumoylation of GCMa in placental cells was studied by transfecting HA-GCMa-expressing BeWo cells with pEGFP-SUMO1 or pEGFP-SUMO1-AA, followed by the above-mentioned detection assay. In separate experiments, HA-GCMa proteins in HA-GCMa-expressing BeWo cells were immunoprecipitated by HA mAb, or the endogenous GCMa proteins in JAR cells were immunoprecipitated by a GCMa antibody (1). The immunoprecipitates were then subjected to immunoblotting with a SUMO1 antibody (Invitrogen). For the in vitro sumoylation assay of GCMa, cytosolic extracts of HeLa cells were prepared according to Kroll et al. (13) and incubated with different combinations of recombinant GCMa-FLAG, Ubc9, GST-SUMO1, and GST-SUMO1-AA at 30 °C for 2.5 h, followed by immunoblotting with FLAG mAb. In a separate experiment, recombinant SUMO E1, SAE1/SAE2 (Boston Biochem, Cambridge, MA), and His-SUMO1 (Boston Biochem) proteins were incubated with HA-GCMa-FLAG and Ubc9 for in vitro sumoylation assay of GCMa.

Mapping of the sumoylation acceptor site in GCMa was first conducted by identification of its minimal domain susceptible to sumoylation. To this end, 293T cells were transfected with the indicated combinations of pGal4-FLAG; pGal4-GCMa-FLAG-(1–436), -(1–220), -(1–300), and -(300–436); pEGFP-SUMO1; and pEGFP-SUMO1-AA as described in the legend to Fig. 3A. The sumoylated domain was detected by immunoprecipitation and immunoblotting with FLAG mAb. The sumoylation acceptor site was further identified in HeLa cells transfected with pEGFP-SUMO1 and wild type pGal4-GCMa-FLAG-(1–300) or its site-specific lysine or glutamic acid mutants, including K149R, K156R, K160R, K3R, and E158A.

Protein Stability, EMSA, and DNA Pull-down Assay—Protein stabilities of HA-GCMa, HA-GCMa-SUMO1, GCMa-FLAG, and SUMO1-GCMa-FLAG were studied by transfecting HeLa cells with pHA-GCMa, pHA-GCMa-SUMO1, pGCMa-FLAG, and pSUMO1-GCMa-FLAG, respectively. 30 h post-transfection, cells were pretreated with 75 μg/ml cycloheximide for 1 h and were harvested immediately or after a continuous treatment of cycloheximide for an additional 1, 2, or 4 h for immunoblotting with HA or FLAG mAb. Band intensities were determined by densitometric analysis using an Eastman Kodak Co. DC290 zoom digital camera and Kodak 1D image analysis software.

Preparation of recombinant MBP fusion proteins has been described previously (2). MBP-GCMa-(1–167) fusion protein contains the minimal domain of GCMa essential for DNA binding. MBP-GCMa-(1–167)-SUMO1 fusion protein is a different version of MBP-GCMa-(1–167) with an additional SUMO1 domain at its C terminus. EMSA experiments using the pGBS oligonucleotide and the prepared MBP fusion proteins were conducted as described previously (1). For the DNA pull-down assay, a biotinylated oligonucleotide containing three copies of the pGBS sense strand sequence was annealed with a complementary oligonucleotide. The annealed oligonucleotide, Biotin-(pGBS)₃, was attached to streptavidin-conjugated magnetic beads according to the manufacturer’s instructions (Polysciences, Warrington, PA). The beads were then incubated with an in vitro sumoylation reaction mixture containing nonsumoylated GCMa-FLAG and His-SUMO1-conjugated GCMa-FLAG in binding conditions similar to EMSA. After extensive washing, the reactions were analyzed by immunoblotting with FLAG mAb. Band intensities were quantified with the Kodak EDAS290 system described above. A previously described wild type pGBS oligonucleotide and its mutant, Mut, were used as competitors in the pull-down assay (1, 14).

Chromatin Immunoprecipitation (ChiP) Assay—Approximately 3 × 10⁶ HeLa cells were mock-transfected or transfected with different combinations of pLUC27950/28314, pLuc(27950/28314)-ΔGBS, pHA-GCMa, pHA-GCMaK156R, pEGFP-SUMO1, and pEGFP-SUMO1-AA. 48 h post-transfection, cells were harvested for ChiP assays to study the effect of sumoylation on in vivo association between GCMa and pGBS in the syncytin promoter as previously described (1). In brief, the associated HA-GCMa-DNA complexes were immunoprecipitated by HA mAb-conjugated agarose beads and PCR-amplified for a specific region containing the pGBS sequence in the syncytin promoter construct. Sequences of primers used for PCR were 5′-CAGTGAACATAGACAGAAGTC-3′ and 5′-TAGAATTGCGGCGGGCCTTTC-3′. PCR products were analyzed on 2% agarose gels.

Protein Modeling—The model for the GCM motif (residues 14–167) of human GCMa protein was constructed by a homology-based modeling method using MODELER version 8.2 (Accelrys, San Diego, CA) based on the 2.9 Å resolution crystal structure of the GCM motif of murine GCMa bound to its octameric DNA target (Protein Data Bank code 1ODH) (15). The GCM motifs of human and murine GCMa proteins share 93.5% amino acid sequence identity, and there are no gaps in the sequence alignment. The coordinates of the SUMO1 molecule were obtained from the 2.1 Å resolution crystal structure of human SUMO1 (residues 19–97) conjugated with thymine DNA glycosylase (Protein Data Bank code 1WYW) (16). Before performing the docking modeling, the structures of the GCM motif and SUMO1 were optimized by energy minimization using CHARMM (Accelrys). The potential energy during minimization was calculated using CHARMM22 force field parameters in a vacuum, and the solvent effect was estimated implicitly using the generalized Born model with smooth switching function for the molecular surface. After 1000 minimization steps using the steepest descent method followed by 1000 steps with adopted basis Newton-Raphson algorithm, the quality of the structures was evaluated by PROCHECK (17). The docking of SUMO1 to GCM motif was conducted by using ZDOCK (18). ZDOCK searched 2000 possible binding modes in the translational and rotational space between the GCM motif and SUMO1 and evaluated each mode by shape complementarity, desolvation free energy, and electrostatics energy. The complex predicted as most probable was chosen as the template structure for making the model of GCM-SUMO1 chimeric protein by energy minimization and the homology-modeling program MODELER. MODELER assigned the coordinates of GCM and SUMO1 domains in the chimeric protein from the GCM-
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SUMO1 complex structure and built the loop structure between the two domains, which originates from the linker sequence (serine-arginine) in the expression plasmid and from the highly disordered N terminus (residues 1–18) of SUMO1. The conformation of the SUMO1 C terminus was altered by XtalView (19) in order to represent the possible SUMO1 conjugation state (i.e. conjugation of glycine 97 of SUMO1 with lysine 156 of the GCM motif). The resulting model structure was further energy-minimized and evaluated by PROCHECK. Molecular visualization was prepared by PyMOL (available on the World Wide Web).

RESULTS

Association of GCMa with Ubc9—To investigate the possibility of GCMa being a substrate for sumoylation machinery, we first examined whether GCMa interacts with Ubc9 and PIAS proteins, two key components for substrate recognition in the sumoylation machinery. 293T cells were transfected with different combinations of pHA-GCMa, pUbc9-FLAG, and pPIAS2β-Myc for co-immunoprecipitation analysis. As shown in Fig. 1A, a specific interaction between GCMa and Ubc9 was observed, whereas no interaction between GCMa and PIAS2β was observed. We also examined the interaction between GCMa and other PIAS proteins: PIAS1, -3, -2α, and -γ. However, these PIASs did not interact with GCMa either (data not shown). To characterize the interaction between GCMa and Ubc9 in vitro, we performed GST pull-down experiments by incubating recombinant GCMa-FLAG protein with recombinant GST or GST-Ubc9 protein. As shown in Fig. 1B, a direct and specific interaction between GCMa and Ubc9 was observed. We further mapped the interaction domains of GCMa for Ubc9 by cotransfecting 293T cells with pUbc9-Myc and a series of pGal4-GCMa-FLAG plasmids encoding different proteins with the Gal4 DNA-binding domain fused with full-length or different regions of the GCMa polypeptide. As shown in Fig. 1C, specific interactions were only detected between Ubc9 and the regions of amino acids 1–436 (full length), 1–220, and 1–300 in GCMa, suggesting that Ubc9 interacts with the N-terminal domain of GCMa. Similar mapping results were also observed in HeLa cells (data not shown). Taken together, these results suggest that GCMa can be recognized by the sumoylation machinery via its Ubc9 component and is therefore highly likely to be a sumoylation substrate.

In Vitro and in Vivo Sumoylation of GCMa—We next investigated whether GCMa can be sumoylated in vivo and in vitro. We performed in vivo sumoylation assays by transfecting 293T cells with different combinations of pHA-GCMa, pEGFP-SUMO1, pEGFP-SUMO1-AA, pUbc9-FLAG, pPIAS2β-Myc, and pPIAS2β-Myc-C/S, followed by immunoprecipitation and immunoblotting with HA mAb. As shown in Fig. 2A, bands corresponding to higher molecular mass GCMa protein was detected when GCMa was coexpressed with EGFP-SUMO1 but not with EGFP-SUMO1-AA, which lacks the terminal Gly-Gly motif for conjugation. Similar results were detected when immunopurified GCMa was immunoblotted with an EGFP mAb, suggesting that the higher molecular mass GCMa is the sumoylated GCMa. The level of sumoylated GCMa was further increased when GCMa was coexpressed with EGFP-SUMO1.
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and Ubc9. In contrast, GCMα sumoylation was inhibited when a dominant negative Ubc9 was expressed in the similar assay conditions (Fig. 2A). Interestingly, another band of higher molecular mass GCMα was visibly detected in the presence of PIAS2/H9252 but not its active site mutant (Fig. 2A), suggesting that PIAS2/H9252 may further promote the degree of GCMα sumoylation.

To test whether GCMα can be sumoylated in placental cells, we first used an HA-GCMα-expressing BeWo stable line for transfection with pEGFP-SUMO1 and pEGFP-SUMO1-AA, respectively. As expected, immunoprecipitation with HA mAb followed by immunoblotting with HA and EGFP mAbs, respectively, showed that HA-GCMα was conjugated with EGFP-SUMO1 but not EGFP-SUMO1-AA (Fig. 2B). We then examined whether the HA-GCMα in the HA-GCMα-expressing BeWo cells and the endogenous GCMα in the human trophoblast JAR cell line can be sumoylated by endogenous SUMO1. Conjugation of HA-GCMα with endogenous SUMO1 was detected in HA-GCMα-expressing BeWo cells subjected to immunoprecipitation using HA mAb and then immunoblotting using a SUMO1 antibody (Fig. 2C, upper panels). As a control, no signals were detected when normal mouse IgG was used for immunoprecipitation. Furthermore, sumoylation of endogenous GCMα was detected in JAR, but not 293T, cells subjected to immunoprecipitation using a GCMα antibody and then immunoblotting using a SUMO1 antibody (Fig. 2C, lower panels).

For in vitro characterization of GCMα sumoylation, recombinant GCMα-FLAG proteins were incubated with HeLa extracts plus different combinations of recombinant Ubc9, GST-SUMO1, and GST-SUMO1-AA. As shown in the left panel of Fig. 2D, Ubc9 promoted sumoylation of GCMα-FLAG only in the presence of GST-SUMO1 and not GST-SUMO1-AA. In a separate experiment, recombinant SUMO E1 (SAE1/SAE2) and His-SUMO1 proteins were used to replace the HeLa extracts and the GST-SUMO1 protein, respectively. As expected, Ubc9 was able to promote sumoylation of GCMα-FLAG in the presence of SAE1/SAE2 (Fig. 2D, right). Taken together, these results suggest that GCMα is a bona fide substrate for sumoylation machinery, and Ubc9 is sufficient to mediate SUMO modification of GCMα in vivo and in vitro.
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To identify the site of sumoylation in GCMa, we performed in vivo sumoylation assays to map the minimal GCMa domain susceptible to sumoylation. To this end, 293T cells were transfected with different combinations of p(pGBS)4E1bLUC, pHA-GCMa, pEGFP-SUMO1, pSENP1-FLAG, and pUbc9-FLAG-dn. As shown in Fig. 4A, the luciferase activity directed by p(pGBS)E1bLUC was activated by GCMa. Moreover, the observed GCMa-mediated transcriptional activation was repressed when pEGFP-SUMO1 was cotransfected. Interestingly, when a SUMO-specific protease, SENP1, or a dominant-negative Ubc9 (Ubc9-dn) was coexpressed with GCMa, the transcriptional activation mediated by GCMa was significantly enhanced (Fig. 4A). To rule out any adversary effect of the bulky EGFP moiety in EGFP-SUMO1 on the observed effect of sumoylation, we also tested pHis-SUMO1 and pHA-SUMO1 in similar transient expression experiments. In fact, similar results were obtained using both constructs (supplemental Fig. 1).

Sumoylation Represses GCMa Transcriptional Activity—Having verified that GCMa is a sumoylation substrate, we further studied the functional consequence of GCMa sumoylation in terms of transcriptional activation. HeLa cells were transfected with 4 μg of pGal4-FLAG or pGal4-GCMa-FLAG or its deletion mutant plus 2 μg of pEGFP-SUMO1 or pEGFP-SUMO1-AA. 48 h post-transfection, cells were harvested for sumoylation analysis as described under “Experimental Procedures.” The asterisks indicate sumoylated wild type or mutant Gal4-GCMa-FLAG. B, identification of the sumoylation acceptor site in GCMa. The lysine residues of three potential sumoylation motifs in GCMa (1–300) are highlighted, and the signature glutamic acid residue in each motif is underlined. HeLa cells were transfected with 2 μg of pEGFP-SUMO1 and 4 μg of wild type (WT) pGal4-GCMa-FLAG (1–300) or its site-specific lysine-arginine or glutamic acid-to-alanine mutant. 48 h post-transfection, cells were harvested for sumoylation analysis as described under “Experimental Procedures.” The arrow indicates sumoylated wild type or mutant Gal4-GCMa-FLAG (1–300).

Identification of the SUMO Acceptor Site in GCMa—Because one major sumoylated GCMa species was detected in the analyses above, we speculated that GCMa might contain a major SUMO acceptor site. To identify this site, we first performed in vivo sumoylation assays to map the minimal GCMa domain susceptible to sumoylation. To this end, 293T cells were transfected with different combinations of pEGFP-SUMO1, pEGFP-SUMO1-AA, pGal4-FLAG, and a series of pGal4-GCMa-FLAG plasmids encoding full-length or deletion mutant GCMa. Sumoylation analysis revealed that sumoylation occurs in the full-length GCMa and the amino acid regions 1–220 and 1–300 of GCMa but not in the Gal4 DNA-binding domain and the amino acid region 300–436 of GCMa (Fig. 3A). Therefore, the minimal GCMa domain susceptible to sumoylation is the N-terminal domain (amino acids 1–220) of GCMa.

Close scrutiny for the SUMO consensus modification motif in the N-terminal domain of GCMa indeed revealed three potential SUMO acceptor sites as lysine 149, lysine 156, and lysine 160 (Fig. 3B). To verify which lysine residue(s) can be sumoylated in vivo, we generated GCMa mutants harboring a single or combined lysine-to-arginine or aspartic acid-to-alanine mutation by site-directed mutagenesis and tested these mutants in sumoylation assays. As shown in Fig. 3B, the mutants (K149R and K160R) harboring the lysine-to-arginine mutation at lysine 149 and lysine 160, respectively, were susceptible to sumoylation. Conversely, no sumoylation was detected in the K156R mutant. Moreover, mutation of the signature glutamic acid in the SUMO consensus modification motif of lysine 156 into alanine (E158A) also eliminated sumoylation on lysine 156 (Fig. 3B). Taken together, these results indicate that lysine 156 is the major SUMO acceptor site in GCMa.

Sumoylation Represses GCMa Transcriptional Activity—Having verified that GCMa is a sumoylation substrate, we further studied the functional consequence of GCMa sumoylation in terms of transcriptional activation. HeLa cells were transfected with different combinations of p(pGBS)E1bLUC, pHA-GCMa, pEGFP-SUMO1, pSENP1-FLAG, and pUbc9-FLAG-dn. As shown in Fig. 4A, the luciferase activity directed by p(pGBS)E1bLUC was activated by GCMa. Moreover, the observed GCMa-mediated transcriptional activation was repressed when pEGFP-SUMO1 was cotransfected. Interestingly, when a SUMO-specific protease, SENP1, or a dominant-negative Ubc9 (Ubc9-dn) was coexpressed with GCMa, the transcriptional activation mediated by GCMa was significantly enhanced (Fig. 4A). To rule out any adversary effect of the bulky EGFP moiety in EGFP-SUMO1 on the observed effect of sumoylation, we also tested pHis-SUMO1 and pHA-SUMO1 in similar transient expression experiments. In fact, similar results were obtained using both constructs (supplemental Fig. 1).

Therefore, sumoylation very likely imposes a repression effect on GCMa transcriptional activity. We were curious about whether mutagenesis of the lysine 156 SUMO consensus modification motif of GCMa affects this repression effect. Therefore, we tested the HA-GCMaK156R and -E158A mutants in transient expression assays. As shown in Fig. 4B, the luciferase activity stimulated by HA-GCMaK156R was higher than that by the wild type HA-GCMa. Although SENP1 further enhancing GCMa activity was consistent with the study above, SENP1 did not significantly affect HA-GCMaK156R activity (p = 0.103 by t test) (Fig. 4B). Because the change of glutamic acid 158 into alanine in HA-GCMaE158A eliminated its DNA binding activity (data not shown), similar functional characterization of this mutant was not further pursued. Nevertheless, our results suggest that sumoylation on lysine 156 represses GCMa transcriptional activity, whereas prevention of sumoylation by Ubc9-dn or desumoylation by SENP1 greatly enhances GCMa transcriptional activity.

We also tested whether sumoylation regulates the promoter activity of syncytin, a GCMa target gene. The GCMa-expressing JAR cell line (supplemental Fig. 2) was transfected with different combinations of p(27950/28314)LUC, p(27950/28314)LUC−ΔGBS, pEGFP-SUMO1, and pUbc9-FLAG-dn. The luciferase activity directed by p(27950/28314)LUC−ΔGBS was much higher than that by p(27950/28314)LUC−ΔGBS, which contains a deletion of the pGBS site (Fig. 4C). This suggests that endogenous GCMa is involved in stimulating the luciferase activity directed by p(27950/28314)LUC. Interestingly, the luciferase activity directed by p(27950/28314)LUC was decreased or increased when pEGFP-SUMO1 or pUbc9-FLAG-dn was cotransfected (Fig. 4C). Conversely, the effects of SUMO1 and dominant negative Ubc9 were less effective on the luciferase activity directed by p(27950/28314)LUC−ΔGBS (Fig. 4C). Taken together, these results suggest that repression of
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Sumoylation decreases the DNA Binding Activity of GCMa—We further investigated the molecular mechanism underlying the repression of GCMa activity by sumoylation. We first tested whether sumoylation affects the half-life of GCMa protein. To mimic GCMa sumoylation, we generated expression plasmids encoding modified GCMa proteins, HA-GCMa-SUMO1 and SUMO1-GCMa-FLAG, with SUMO1 linking to the C-terminal of HA-GCMa and to the N-terminal of GCMa-FLAG, respectively. Individual expression constructs for HA-GCMa, HA-GCMa-SUMO1, GCMa-FLAG, and SUMO1-GCMa-FLAG were transfected into HeLa cells, followed by treatment without or with cycloheximide for different time periods in order to determine their protein stabilities. As shown in Fig. 5A, the half-lives for each protein were not significantly different from each other, with values around 90 min after linear regression analysis. In addition, we also compared protein stabilities of HA-GCMa and HA-GCMaK156R in HeLa cells. We observed comparable levels of both proteins in HeLa cells transfected with different amounts of expression plasmids (supplemental Fig. 3). Moreover, the half-lives for both proteins were not significantly different from each other (supplemental Fig. 3). Therefore, sumoylation is unlikely to change or shorten the half-life of GCMa. On the other hand, we also tested whether sumoylation affects the cellular localization of GCMa by immunohistochemistry in 293T, BeWo, and HeLa cells transfected with pHA-GCMa and pEGFP-SUMO1. Coexpression of SUMO1 with GCMa did not significantly change the nuclear distribution of GCMa (supplemental Fig. 4).

Because lysine 156 is within the DNA-binding domain of GCMa (amino acids 1–167), we were curious whether sumoylation affects the DNA binding activity of GCMa. We therefore prepared recombinant MBP-GCMa-(1–167) and MBP-GCMa-(1–167)-SUMO1 for EMSA analysis using an oligonucleotide (pGBS) derived from the proximal GCMa-binding site in the syncytin promoter as probe. To compare the binding efficiency of both proteins, different amounts of unlabeled pGBS oligonucleotide were used as competitors in the analysis. Unlabeled pGBS at a 5-fold molar excess over the probe almost completely blocked the interaction between labeled pGBS and MBP-GCMa-(1–167)-SUMO1 (Fig. 5B, right). Interestingly, the interaction between labeled pGBS and MBP-GCMa-(1–167) was relatively stable in the presence of this amount of unlabeled pGBS (Fig. 5B, left), showing that the binding efficiency of MBP-GCMa-(1–167)-SUMO1 to pGBS was lower than that of MBP-GCMa-(1–167). In a separate study using a mutant MBP-GCMa-(1–167) harboring the K156R mutation, the DNA binding activity of this mutant was not significantly different from that of wild type (data not shown).

Because recombinant GCMa-FLAG proteins can be sumoylated in the presence of recombinant SUMO E1, Ubc9, and His-SUMO1 proteins (Fig. 2D), we further tested the DNA binding activity of sumoylated GCMa in vitro. To this end, biotinylated pGBS fragments, Biotin-(pGBS)₃, were prepared and attached to streptavidin-conjugated magnetic beads for a DNA pull-down assay of nonsumoylated and His-SUMO1-conjugated GCMa-FLAG. We first determined the optimal amount of GCMa-FLAG for the assay and found that 50 ng of GCMa-FLAG proteins is pertinent, because all of the input GCMa-FLAG proteins bound to Biotin-(pGBS)₃, and no free-form GCMa-FLAG proteins were detected in the supernatants after reaction (Fig. 5C, top). In addition, spe-
Specific interaction between GCMa-FLAG and Biotin-(pGBS)₃ was observed because a wild type pGBS oligonucleotide, but not its mutant, Mut, competed out the association between GCMa-FLAG and Biotin-(pGBS)₃ (Fig. 5C, middle). To test whether sumoylated GCMa-FLAG binds to pGBS, we performed in vitro sumoylation reactions with GCMa-FLAG,
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Ubc9, and His-SUMO1 in the presence or absence of SUMO E1. Based on densitometric analysis, 17.5% of the input GCMa-FLAG was conjugated with His-SUMO1 in the reaction containing SUMO E1 (Fig. 5C, bottom). When this reaction mixture was incubated with Biotin-(pGBS), the retrieved His-SUMO1-conjugated GCMa-FLAG only occupied 1.1% of the total retrieved GCMa-FLAG (Fig. 5C, bottom). Because the level of retrieved nonsumoylated GCMa-FLAG was comparable with that of the input nonsumoylated GCMa-FLAG, these results indicate that sumoylated GCMa-FLAG was not proportionally pulled down and suggest that sumoylated GCMa-FLAG has a decreased DNA binding activity.

To confirm the aforementioned inhibitory effect of sumoylation in vivo, we performed ChIP assays in HeLa cells transfected with different combinations of p(27950/28314)LUC, p(27950/28314)LUC-ΔGBS, pHA-GCMa, pEGFP-SUMO1, and pEGFP-SUMO1-AA. As shown in the upper panel of Fig. 5D, specific interaction between HA-GCMa and the pGBS site in p(27950/28314)LUC was detected. Interestingly, this interaction was significantly reduced in the presence of EGFP-SUMO1 but not EGFP-SUMO1-AA. As a control, no interaction between HA-GCMa and the pGBS-deleted syncytin promoter in the p(27950/28314)LUC-ΔGBS was detected. More importantly, the decrease in the interaction between HA-GCMa and pGBS may be attributed to the effect of sumoylation on lysine 156, because the interaction between HA-GCMaK156R and pGBS was not affected in the presence of EGFP-SUMO1 (Fig. 5D, bottom). Taken together, these results suggest that sumoylation on lysine 156 is highly likely to reduce the DNA-binding activity of GCMa and therefore repress GCMa-mediated transcriptional activation.

Because the three-dimensional structures of GCM motif and SUMO1 are available, we further performed molecular modeling to explore the molecular mechanism underlying the reduced DNA-binding activity of the GCM motif caused by sumoylation. By docking SUMO1 to the GCM motif and using energy minimization, the top structures of GCM-SUMO1 that were predicted to be most probable consistently revealed that the space of the SUMO1 moiety in the SUMO1-conjugated GCM motif partially overlaps with the DNA-binding surfaces of GCM motif (Fig. 6, A and B). The lysine 156, positioned in the loop between the β-sheet 7 and the helix 3 of GCM motif (Fig. 6A), is highly accessible to conjugation with the glycine 97 of SUMO1. These results, in concert with the results of EMSA and DNA pull-down assays above, suggest that SUMO1 modification at lysine 156 imposes steric hindrance on the DNA-binding surfaces of the GCM motif and thereby reduces its DNA binding activity.

DISCUSSION

GCMa plays a pivotal role in the regulation of placental development (20, 21). Specifically, GCMa can regulate the differentiation, via cell-cell fusion, of mononucleated cytotrophoblasts into a multinucleated syncytiotrophoblast layer that covers the surface of a placental villus. In this process, GCMa up-regulates the expression of syncytin, a fusogenic protein executing the cell-cell fusion event (1). To maintain the physiological integrity of the placenta, the protein level of syncytin and hence GCMa activity needs to be well controlled. It is feasible that GCMa activity is regulated at multiple levels. Indeed, GCMa is a phosphoprotein, and its protein level can be post-translationally regulated by protein ubiquitination and acetylation (2, 4). In the present study, we provide evidence that sumoylation negatively regulates GCMa activity by decreasing its DNA binding activity. We demonstrated that Ubc9, the E2 subunit of sumoylation machinery, directly interacts with GCMa at its N-terminal GCM motif. We also demonstrated that GCMa can undergo SUMO modification and identified lysine 156 as the major SUMO acceptor site in vivo. Furthermore, GCMa sumoylation represses its transcriptional activity, most likely by blocking the interaction between the GCM motif and its cognate DNA element. In terms of physiological context, we also demonstrated that GCMa-mediated syncytin promoter activity was repressed or enhanced in the presence of a high level of SUMO1 or dominant-negative Ubc9 protein in placental cells.

Although several lysine residues and their flanking sequences in GCMa are similar to the SUMO consensus sequence, PKXE, we identified the lysine 156 SUMO sequence, PKPE, as the major SUMO acceptor site in GCMa. This observation is sup-
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ported by the major SUMO1-modified GCM\(a\) species detected by in vivo and in vitro sumoylation assays. In addition, a change of glutamic acid 158 downstream of lysine 156 into alanine abolished GCM\(a\) sumoylation, strongly suggesting that lysine 156 is a functional SUMO acceptor site in GCM\(a\). Of note, a proline residue occurs just before lysine 156, which may not match the requirement for a large hydrophobic amino acid in the SUMO consensus sequence. However, noncanonical sumoylation sites with sequences quite different from the SUMO consensus sequence have been identified in several proteins, such as PML, which has an AKCP sequence (22), and Smad4, which has a VKYC sequence (23). Given that Ubc9 directly recognizes the PKXE sequence and extensive interactions between Ubc9 and sequences outside this SUMO consensus motif have been reported in the RanGAP1-Ubc9 complex (7), structural flexibilities or E3 proteins may facilitate the interaction between Ubc9 and its target proteins to accommodate variations in the lysine 156 SUMO sequence of GCM\(a\) and in the reported noncanonical sumoylation sequences. On the other hand, the possibility of minor SUMO acceptor site(s) in GCM\(a\) cannot be ruled out, because an additional sumoylated form of GCM\(a\) was observed in the presence of the SUMO E3 ligase, Pias2\(B\). In the Smad4 sumoylation studies, interestingly, one study only identified lysine 159 as the SUMO acceptor site in Smad4 (24), whereas the two other studies identified lysine 113 as an additional SUMO site (23, 25). The reasons why different conclusions were reached are not clear; however, different assay conditions (e.g. different cell lines were studied) may have led to different observations in these studies. In this regard, identification of the minor SUMO site(s) in GCM\(a\) may be facilitated by different or more sensitive assay conditions.

In the present study, repression of GCM\(a\) transcriptional activity by sumoylation on lysine 156 was further attributed to inhibition of its DNA binding activity. Based on the available three-dimensional structures of the GCM-DNA complex and SUMO1, we performed protein modeling using the GCM\(a\)-(1–167)-SUMO1 chimeric protein to address this functional outcome of GCM\(a\) sumoylation. In our model, the SUMO1 moiety in the GCM-SUMO1 complex partially overlaps with the DNA-binding surfaces of the GCM motif. Although the GCM\(a\)-(1–167)-SUMO1 chimeric protein was constructed with the N terminus of SUMO1 fused with the C terminus of the GCM motif, we believe that this chimeric protein is able to mimic SUMO1 modification at lysine 156. Specifically, this is supported by the fact that fusion of the highly disordered N terminus (residues 1–18) of SUMO1 with the C terminus of GCM motif still provided a flexible structure to allow a close interaction between SUMO1 and the GCM motif such that the formation of an isopeptide bond between glycine 97 of SUMO1 and lysine 156 of GCM motif is attainable (Fig. 6A). Therefore, the structural model derived from the GCM\(a\)-(1–167)-SUMO1 chimeric protein is highly likely to represent the conformation of the GCM-SUMO1 complex. Sumoylation of several DNA-binding proteins, including HSF2, TDG, and the TFIIID subunit, TAF5, has been shown to decrease their DNA binding ability. Sumoylation on lysine 82 in HSF2 is regulated by a loop in its DNA-binding domain, which results in inhibition of the DNA binding activity of HSF2 (26). Similarly, sumoylation on lysine 14 in TAF5 prevents TFIIID binding to promoter DNA (27). Whether sumoylation on HSF2 and TAF5 causes conformational changes inhibiting their DNA binding activities is still an open question. Interestingly, sumoylation on lysine 330 in the uracil/thymine DNA glycosylase TDG induces a conformational change by forming a protruded helix, which causes a steric clash with the DNA backbone (16). Based on our EMSA, DNA pull-down, and ChIP assays and modeling analysis, here we propose that the partial overlapped surfaces in GCM\(a\) for conjugated SUMO1 and bound DNA may also cause a steric clash that destabilizes the interaction between GCM\(a\) and its cognate DNA sequence.

Syncytin-mediated cytotrophoblastic fusion is important for syncytiotrophoblast formation. Our previous studies have indicated that regulation of this fusion can be achieved by the cytoplasmic domain of syncytin (28) and by GCM\(a\)-mediated syncytin gene expression (1) as well as by post-translational modifications of GCM\(a\), such as acetylation and ubiquitination, that increase and decrease the protein level of GCM\(a\), respectively (2, 4). In the present study, we further demonstrated that GCM\(a\) activity can be regulated by sumoylation on lysine 156 in its GCM motif, resulting in decreased DNA binding activity. Therefore, GCM\(a\) sumoylation provides an additional measure to down-regulate GCM\(a\) activity in the placenta and may therefore help to maintain the physiological functions of the syncytiotrophoblast layer.

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REFERENCES

1. Yu, C., Shen, K., Lin, M., Chen, P., Lin, C., Chang, G. D., and Chen, H. (2002) J. Biol. Chem. 277, 50062–50068
2. Chang, C. W., Chuang, H. C., Yu, C., Yao, T. P., and Chen, H. (2005) Mol. Cell. Biol. 25, 8401–8414
3. Chuang, H. C., Chang, C. W., Chang, G. D., Yao, T. P., and Chen, H. (2006) Nucleic Acids Res. 34, 1459–1469
4. Yang, C. S., Yu, C., Chuang, H. C., Chang, C. W., Chang, G. D., Yao, T. P., and Chen, H. (2005) J. Biol. Chem. 280, 10083–10090
5. Melchior, F. (2000) Annu. Rev. Cell Dev. Biol. 16, 591–626
6. Johnson, E. S. (2004) Annu. Rev. Biochem. 73, 355–382
7. Bernier-Villamor, V., Sampson, D. A., Matunis, M. J., and Lima, C. D. (2002) Cell 108, 345–356
8. Lee, H., Quinn, J. C., Prasanth, K. V., Swiss, V. A., Economides, K. D., Camacho, M. M., Spector, D. L., and Abate-Shen, C. (2006) Genes Dev. 20, 784–794
9. Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., and Hay, R. T. (2003) Mol. Cell 11, 1043–1054
10. Yang, S. H., and Sharrocks, A. D. (2004) Mol. Cell 13, 611–617
11. Spengler, M. L., and Brattain, M. G. (2006) J. Biol. Chem. 281, 5567–5574
12. Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., and Sistonen, L. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 45–50
13. Kroll, M., Conconi, M., Desterro, M. J., Marin, A., Thomas, D., Friguet, B., Hay, R. T., Virelizier, J. L., Arenzana-Seisdedos, F., and Rodriguez, M. S. (1997) Oncogene 15, 1841–1850
14. Schreiber, J., Enderich, J., and Wegner, M. (1998) Nucleic Acids Res. 26, 2337–2343
15. Cohen, S. X., Moulin, M., Hashemolhosseini, S., Kilian, K., Wegner, M.,
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22. Kamitani, T., Kito, K., Nguyen, H. P., Wada, H., Fukuda-Kamitani, T., and Yeh, E. T. (1998) J. Biol. Chem. 273, 26675–26682
23. Lin, X., Liang, M., Liang, Y. Y., Brunicardi, F. C., Melchior, F., and Feng, X. H. (2003) J. Biol. Chem. 278, 18714–18719
24. Ohshima, T., and Shimotohno, K. (2003) J. Biol. Chem. 278, 50833–50842
25. Lee, P. S., Chang, C., Liu, D., and Derynck, R. (2003) J. Biol. Chem. 278, 27853–27863
26. Anckar, J., Hietakangas, V., Denessiouk, K., Thiele, D. J., Johnson, M. S., and Sistonen, L. (2006) Mol. Cell. Biol. 26, 955–964
27. Boyer-Guittaut, M., Birsoy, K., Potel, C., Elliott, G., Jaffray, E., Desterro, J. M., Hay, R. T., and Oelgeschlager, T. (2005) J. Biol. Chem. 280, 9937–9945
28. Chang, C., Chen, P. T., Chang, G. D., Huang, C. J., and Chen, H. (2004) Biol. Reprod. 71, 1956–1962