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Stimulation of GCMa Transcriptional Activity by Cyclic AMP/Protein Kinase A Signaling Is Attributed to CBP-Mediated Acetylation of GCMa†

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Human GCMa is a zinc-containing transcription factor primarily expressed in placenta. GCMa regulates expression of *syncytin* gene, which encodes for a placenta-specific membrane protein that mediates trophoblastic fusion and the formation of syncytiotrophoblast layer required for efficient fetal-maternal exchange of nutrients and oxygen. The adenylate cyclase activator, forskolin, stimulates *syncytin* gene expression and cell fusion in cultured placental cells. Here we present evidence that cyclic AMP (cAMP) signaling pathway activates the *syncytin* gene expression by regulating GCMa activity. We found that forskolin and protein kinase A (PKA) enhances GCMa-mediated transcriptional activation. Furthermore, PKA treatment stimulates the association of GCMa with CBP and increases GCMa acetylation. CBP primarily acetylates GCMa at lysine[367], lysine[406], and lysine[409] in the transactivation domain (TAD). We found that acetylation of these residues is required to protect GCMa from ubiquitination and increases the TAD stability with a concomitant increase in transcriptional activity, supporting the importance of acetylation in PKA-dependent GCMa activation. Our results reveal a novel regulation of GCMa activity by cAMP-dependent protein acetylation and provide a molecular mechanism by which cAMP signaling regulates trophoblastic fusion.

GCM1 (*glial cell missing*), also named *Glide* (*Glial cell deficient*), was first isolated from a *Drosophila melanogaster* mutant line that produces additional neurons at the expense of glial cells. Conversely, ectopic expression of *GCM1* in flies generated excessive numbers of glial cells at the expense of neurons (24, 26). It is thought that GCM functions as a genetic binary switch between neuronal and glial determination in *Drosophila*. *Drosophila* GCM1 is transiently expressed in glial precursors and immature glial cells except for mesectodermal midline glia in the central nervous system and many of the specialized support cells of PNS sensory neurons (24, 26). Recently, a GCM1 homologue called *GCM2* or *Glide2*, located 27 kb apart from the *GCM1* locus in the *Drosophila* genome, was isolated (2, 27). GCM2 has redundant functions of GCM1 and plays a minor role during gliogenesis. However, both GCM1 and -2 are required for the proper differentiation of the plasmatocyte/macrophage lineage of blood cells (2).

Two GCM homologues called *GCMa* and *b* have been identified in mice, rats, and humans (28, 30). In contrast to the neural expression pattern of *Drosophila* GCM1 and -2, mouse GCMa mRNA is highly expressed in the labyrinthine trophoblast cells of placenta and at low levels in restricted sites of the postnatal kidney and thymus (4, 21). GCMa is required for placental development because genetic ablation of mouse *GCMa* leads to failure of labyrinth layer formation and no fusion of trophoblasts to syncytiotrophoblasts (3, 37). *GCMb* is required for the proper development of parathyroid glands (19). Recently, chicken *GCM* has been isolated and shown to be exclusively expressed in extra-embryonic tissues (22). Since mammalian *GCMa* is also expressed in extra-embryonic tissues, it has been speculated that GCM evolutionary function is conserved between mammals and birds. In addition, zebra fish *GCMb* has been characterized and shown to be required for normal development of pharyngeal cartilages (20, 23).

GCM proteins form a novel family of transcription factor with a conserved DNA-binding domain, termed the GCM motif, at the N terminus (1, 36). Recent crystallographic analysis of the GCM motif has revealed that it is a zinc-containing domain of β sheets interacting with the major groove of its cognate DNA element, 5′-ATGCGGGT-3′ (14). Transactivation domain has been identified in the carboxyl terminus of GCM proteins (1, 38). In terms of physiological function, *Drosophila* GCM1 regulates expression of *repo* (*reverse polarity*) and *pnt* (*pointed*) genes, the principal mediators of glial differentiation, whereas human GCMa regulates expression of the *syncytin* gene, which encodes a placental fusogenic membrane protein mediating trophoblastic fusion (39, 45). Syncytin is an envelope (Env) protein of the newly identified human endogenous retrovirus family W (HERV-W), which is a class I HERV with sequences homologous to the mammalian type C retroviruses and a tRNA primer-binding site for $tRNA^{Trp}$ (7, 33). Two functional GCMa-binding sites in the 5′-flanking region of the 5′ long terminal repeat (LTR) of the HERV-W have been identified (45). This suggests that GCMa regulates syncytin-mediated trophoblastic fusion at the transcriptional level. Like other retroviral Env proteins, syncytin is posttranslationally cleaved into a surface (SU) subunit and a transmem-

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brane (TM) subunit, which contains a fusion peptide. Two sodium-dependent amino acid transporters, ASCT1 and -2, have been reported as the syncytin receptors (32). It is generally believed that syncytin binds to its cognate receptor via its SU subunit and results in a conformational rearrangement in its TM subunit in the fusion process. Indeed, our recent study has demonstrated that interaction between two heptad repeat regions in the TM subunit is required for syncytin-mediated cell fusion (10). It is feasible to speculate that this interaction facilitates exposure and insertion of the fusion peptide into the target cell membrane.

It has been shown that treatment of human placental cells with the adenylate cyclase activator, forskolin, dramatically increases cell-cell fusion (29, 40). In addition, the mRNA level of syncytin in placental BeWo cells is increased after forskolin stimulation (33). These observations suggest that the cAMP/PKA signaling pathway is involved in the syncytin-mediated cell fusion and prompted us to investigate whether GCMa activity is regulated by the cAMP/PKA signaling pathway. In the present study, we demonstrate that forskolin and protein kinase A (PKA) stimulate GCMa-mediated transcriptional activation and CBP is involved in this pathway by directly interacting with and acetylating GCMa. Moreover, PKA facilitates the interaction between CBP and GCMa to promote GCMa acetylation, which increases the protein stability of GCMa and enhances GCMa-mediated transcriptional activation. Our studies help to show how the forskolin-activated cyclic AMP (cAMP)/PKA signaling pathway regulates trophoblastic fusion at the molecular level.

MATERIALS AND METHODS

Plasmid constructs. The pHA-GCMa expression plasmid was constructed by cloning into the pEF1-MycHis expression plasmid (Invitrogen, Carlsbad, CA), a DNA fragment encoding human GCMa with a triple hemagglutinin (HA) tag at its N terminus. The pGCMa-Myc expression plasmid was similar to pHA-GCMa except that it contained a quadruple Myc tag attached to the C terminus of GCMa. pPKAcata was constructed by cloning into pRcCMV (Invitrogen), a DNA fragment encoding the catalytic subunit of the cAMP-dependent PKA. pPKI and pRevAB constructs encoding a peptide inhibitor specific to PKAcata and a dominant-negative regulatory subunit of PKA, respectively, were kindly provided by Stanley McKnight (University of Washington, Seattle). Four tandem copies of the proximal GCMa-binding site (pGBS, 5′-TTCTGGGATGAGGGC AAAACG-3′) in the 5′-LTR of the *syncytin*-containing HERV-W was cloned into pE1bCAT, which harbors a minimal promoter element from the adenovirus *E1B* gene and the bacterial CAT coding sequence, to generate the reporter construct, $p(pGSB)_4$E1bCAT. $p(pGBS)_4$E1bLUC was similar to $p(pGBS)_4$ E1bCAT except that its CAT reporter gene was replaced with a firefly *luciferase* gene. $p(Mut)_5$E1bLUC contained five tandem copies of a mutant pGBS (Mut, 5′-TTCTGGGATGAT*A*GCAAAACG-3′, which is not recognized by GCMa) in pE1bLUC. pLUC(25468-30953) reporter construct containing the *syncytin* promoter element was similar to pCAT(25468-30953) described previously (45), except that its *CAT* reporter gene was replaced with a firefly *luciferase* gene. pG5LUC, a luciferase reporter plasmid containing five tandem copies of a GAL4 binding site, was obtained from Promega (Madison, WI). The pCBP-HA and pCBP-Flag expression plasmids encoded mouse CBP with a C-terminal HA and FLAG tag, respectively, under the control of a cytomegalovirus (CMV) enhancer and promoter. $pCBP^{HAT-}$-HA is a mutant pCBP-HA harboring leucine^{1690}-to-lysine and cysteine^{1691}-to-leucine mutations, which cause loss of the intrinsic histone acetyltransferase (HAT) activity of CBP (41). $pCBP^{HAT-}$-Flag was similar to $pCBP^{HAT-}$-HA except that its HA tag was replaced with a FLAG tag. The pHA-EGFP expression plasmid was constructed by cloning into pEF1-MycHis a DNA fragment encoding EGFP with a triple HA tag at its N terminus. A pGal4-Flag expression plasmid was constructed by cloning the GAL4 DNA-binding domain into p3XFLAG-CMV14 (Sigma, St. Louis, MO). Full-length GCMa and truncated GCMa cDNAs were subcloned into pGal4-Flag to generate wild-type and mutant pGal4-GCMa-Flag expression plasmids, as indicated in the legend to Fig. 7A. Site-directed mutagenesis of the lysine residues in pGal4-GCMa-Flag(300-436) was performed by two-step PCRs with designated primer pairs to generate K349R, K367R, K406R, K409R, K2R, and K3R constructs. The K2R construct is a mutant pGal4-GCMa-Flag(300-436) harboring lysine to arginine mutations in lysine^{406} and lysine^{409}. The K3R construct harbors lysine to arginine mutations in lysine^{367}, lysine^{406}, and lysine^{409}. Similar strategies were used to generate full-length mutant GCMa expression plasmids pGCMa-Myc-K349R, -K2R, and -K3R. All constructs were verified by DNA sequencing by using the dideoxy chain termination method.

Cell culture, transfection, reporter gene assay, and RNA interference. The human trophoblast cell lines, BeWo and JAR, were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained at 37°C in F-12K medium supplemented with 15% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml). Stable BeWo cells expressing HA-GCMa described previously (45) were maintained in the same culture conditions for BeWo cells. 293T and CV1 (ATCC) were maintained at 37°C in HEPES-buffered Dulbecco modified minimal essential medium (DMEM) supplemented with 10% FBS and the same antibiotics mentioned above. For transient expression, 293T and JAR cells were incubated with calcium phosphate-DNA coprecipitates containing the indicated amounts of reporter plasmid and expression plasmid as described in the figure legends. Adjusted amounts of the empty expression vector were also included to maintain a constant amount of total DNA in each transfection assay. Transfection of BeWo and CV1 cells was performed by using the TransIT LT1 reagent (Mirus, Madison, WI) according to the instructions of the manufacturer. Cells were harvested in the reporter lysis buffer (Promega) 48 h posttransfection. CAT and luciferase assays were performed as previously described (11). Specific CAT and luciferase activities were normalized by protein concentration. Protein concentrations were measured by using the BCA protein assay kit (Pierce, Rockford, IL). For RNA interference, 293T cells were transfected with CBP small interfering RNA (siRNA; Santa Cruz Biotechnology, Santa Cruz, CA) or GL2 siRNA (Dharmacon Research, Lafayette, CO) by using the TransIT TKO reagent (Mirus), followed by transfection with the indicated reporter and expression plasmids by using the TransIT LT1 reagent. The efficacy of CBP siRNA was verified in 293T cells by reverse transcriptase-PCR (RT-PCR) with RNeasy reagents (QIAGEN, Hilden, Germany) for RNA purification and the SuperScript III first-strand synthesis system (Invitrogen) for first-strand cDNA synthesis. The sequences of the primers were 5′-ACCTTAGACCCCGAA C-3′ and 5′-CCGTGACTTCATCCCG-3′ for *CBP* and 5′-CTCAAGGGCATCCT GGGCTA-3′ and 5′-CTGTTGCTGTAGCCAAATTCGTT-3′ for *GAPDH*.

Coimmunoprecipitation and pull-down assay. To study the interaction between GCMa and CBP, 293T cells were cotransfected with the indicated combinations of pCBP-HA, pHA-GCMa, and pPKAcata as described in the figure legend. At 48 h posttransfection, cells were harvested in lysis buffer containing 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. Approximately 180 μg of cell lysate was immunoprecipitated with GCMa antibody and protein A-conjugated agarose beads (Roche, Mannheim, Germany). After extensive washing, the immune complexes were analyzed by immunoblotting with a rat monoclonal anti-HA antibody (HA monoclonal antibody [MAb]; Roche). The interaction between GCMa and CBP in placental cells were analyzed in stable HA-GCMa-expressing BeWo cells by the above-mentioned coimmunoprecipitation assay using HA MAb and CBP antibodies from different sources, including A-22 (Santa Cruz Biotechnology), a rabbit polyclonal anti-CBP antibody (Upstate, Lake Placid, NY), and AC26 (CBP MAb) (15). To specify the functional role of PKA in the interaction between GCMa and CBP, HA-GCMa or CBP-HA was first immunoprecipitated from 293T cells cotransfected with pHA-GCMa and pPKAcata or pCBP-HA and pPKAcata. Subsequently, the immune complexes were treated with 200 U of lambda protein phosphatase (λ-PPase; NEB, Beverly, MA) with or without 20 mM Na_3VO_4 at 30°C for 1 h, followed by incubation with 1 μg of recombinant Flag-CBP or GCMa-Flag proteins in the lysis buffer at 4°C overnight. After extensive washing, the pull-down complexes were analyzed by immunoblotting with a mouse monoclonal anti-FLAG antibody (FLAG MAb; Sigma). Recombinant Flag-CBP and GCMa-Flag proteins were purified from Sf9 cells infected with recombinant baculovirus strains using the anti-FLAG M2-conjugated agarose beads (Sigma).

The glutathione *S*-transferase (GST) fusion protein expression vector pGEX4T-1 (Amersham Biosciences, Piscataway, NJ) was used to express GST fusion proteins of full-length GCMa, GST-GCMa(1-436), and truncated GCMa, GST-GCMa(1-220), -GCMa(1-300), -GCMa(1-349), -GCMa(167-349), -GCMa (220-330), -GCMa(300-436), and -GCMa(349-436) in the *Escherichia coli* strain BL21(DE3). Purification of GST fusion proteins was performed as described by Frangioni and Neel (16). For GST-CBP fusion proteins, the corresponding

mouse CBP cDNA fragments were cloned into the pGEX6P-1 vector (Amersham Biosciences) to express GST-CBP(1-451), -CBP(451-721), -CBP(721-1100), -CBP(1099-1460), -CBP(1460-1891), -CBP(1892-2163), and -CBP(2114-2441) fusion proteins. To map the interacting domains of GCMa and CBP, GST pull-down assays were performed. In brief, cell lysates were prepared from 293T cells transfected with pCBP-HA and pHA-GCMa, respectively. Per reaction, 180 μg of the indicated cell lysate was incubated with 2.5 μg of the indicated GST fusion protein prebound in the glutathione beads (Amersham Biosciences) at 4°C overnight. After extensive washing, the pull-down complexes were analyzed by immunoblotting with HA MAb.

ChIP assay. To study association of GCMa and CBP with the promoter region of *syncytin* gene, 3×10^6 BeWo cells were treated with or without 50 μM forskolin for 12 h and analyzed by chromatin immunoprecipitation (ChIP) assays as described by Boyd and Farnham (8). Associated protein-DNA complexes were incubated with GCMa antibody or the CBP antibody from Upstate and then precipitated with protein A-conjugated agarose beads. A specific region containing the pGBS sequence in the syncytin promoter in the immune complexes was detected by PCR with specific primers. PCR conditions included denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 50 s for 40 cycles. PCR products were analyzed on 5% polyacrylamide gels. Sequences of primers were 5′-CTCTCTGGAGAGTGAATTACTGAGTC-3′ and 5′-CCTGTCTCTCAGTTGCAAGATAATTGC-3′ for *syncytin* and 5′-AAAAGCGGGGAGAAAGTAGG-3′ and 5′-CTAGCCTCCCGGGTTTCTCT-3′ for *GAPDH*.

RNA was isolated from BeWo cells treated with or without forskolin using RNeasy reagents, and the syncytin and β-actin transcripts were analyzed by Northern blotting with full-length syncytin and β-actin cDNAs as probes.

In vitro acetylation study and EMSA. For recombinant MBP-GCMa fusion proteins, GCMa cDNA was cloned into the pMAL-c2 vector (NEB) and expressed in BL21(DE3). Affinity purification of MBP-GCMa proteins was performed with maltose agarose beads (NEB) according to the manufacturer's instructions. For in vitro acetylation of GCMa, 1.5 μg of MBP or MBP-GCMa protein was incubated with 150 ng of the purified recombinant Flag-CBP proteins in a 30 μl of reaction buffer (50 mM HEPES [pH 8.0], 10 mM sodium butyrate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) plus or minus 0.8 mM acetyl coenzyme A (Ac-CoA) at 30°C for 1 h. For acetylation analysis, the reaction mixture was analyzed by immunoblotting with a mouse monoclonal anti-acetylated-lysine antibody (Ac-K MAb; Cell Signaling, Beverly, MA). Immunoblotting of MBP and MBP-GCMa proteins was performed with a mouse monoclonal anti-MBP antibody (MBP MAb; Clontech, Palo Alto, CA). For electrophoretic mobility shift assay (EMSA), unacetylated or acetylated MBP-GCMa was incubated with a ^{32}P-labeled pGBS oligonucleotide probe as previously described (45).

In vivo acetylation study and pulse-chase analysis of protein turnover. To study CBP-mediated acetylation of GCMa in vivo, 293T cells were transfected with pGal4-GCMa-Flag alone or with the indicated combinations of pGal4-GCMa-Flag, pCBP-HA, pCBP^{HAT-}-HA, and pPKAcata. The pHA-EGFP expression plasmid was included in each transfection group as an internal control of transfection efficiency. At 48 h posttransfection, cells were harvested for immunoprecipitation with GCMa Ab. The immune complexes were further analyzed by immunoblotting with Ac-K MAb. To study the acetylation of GCMa in placental cells, stable HA-GCMa-expressing BeWo cells were mock treated, treated with forskolin alone, or treated with forskolin together with trichostatin A (TSA) for 24 h, followed by acetylation analysis with HA MAb for immunoprecipitation and Ac-K MAb for immunoblotting. To study the effect of acetylation on protein stability of GCMa, 293T cells in 10-cm culture dishes were transfected with the indicated combinations of pHA-GCMa, pCBP-Flag, and pPKAcata. At 18 h posttransfection, cells were subcultured into 3.5-cm culture dishes for pulse-chase experiments. At 36 h posttransfection, cells were pulse-labeled with 50 μCi of [^{35}S]methionine/ml for 2 h. After labeling, cells were washed twice with phosphate-buffered saline and incubated in chase medium (DMEM with 10% FBS plus 50 μg of methionine/ml) for various time periods. Radiolabeled HA-GCMa proteins were immunoprecipitated with the HA MAb and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Quantification of pulse-chase experiments was performed by using the bioimaging analyzer BAS-1500 (Fujifilm, Kanagawa, Japan). To study the effect of acetylation on GCMa ubiquitination, 293T cells were transfected with different combinations of pHA-Ub, pGCMa-Myc, lysine-to-arginine mutant pGCMa-Myc, pCBP-Flag, pCBP^{HAT-}-Flag, and pPKAcata, followed by immunoprecipitation with a mouse monoclonal anti-Myc antibody (Myc MAb; Roche) and immunoblotting with HA MAb.

Mapping of CBP acetylation sites in GCMa. To map the acetylation domains in GCMa in vitro, 0.2 μg of GST or the indicated GST-GCMa fusion protein was incubated with 0.4 μg of Flag-CBP under the same reaction conditions as described for in vitro acetylation analysis. To identify the acetylation domain in GCMa in vivo, 293T cells were cotransfected with pCBP-HA and pPKAcata and a series of pGal4-GCMa-Flag expression plasmids containing truncated regions of GCMa. Gal4-GCMa-Flag proteins were immunoprecipitated with GCMa Ab and analyzed for acetylation as described above. To identify the CBP acetylation sites in the C-terminal TAD of GCMa, 293T cells were cotransfected with pCBP-HA, pPKAcata, and the wild-type or mutant pGal4-GCMa-Flag(300-436) expression plasmids, followed by acetylation analysis. Similar acetlyation site analyses of full-length GCMa were also performed using pGCMa-Myc and lysine-to-arginine mutant pGCMa-Myc.

RESULTS

Forskolin regulates the transcriptional activity of GCMa. Since *syncytin* is a target gene of GCMa and its expression is stimulated by the adenylate cyclase activator, forskolin, we tested whether forskolin could also stimulate the transcriptional activity of GCMa. The p(pGBS)$_4$E1bCAT reporter plasmid containing four copies of the proximal GCMa-binding site (pGBS) in the promoter region of *syncytin* was constructed. In transient-expression experiments, 293T cells, which lack endogenous GCMa, were transfected with p(pGBS)$_4$E1bCAT alone or plus the GCMa expression plasmid pHA-GCMa and then further treated with or without forskolin. As shown in Fig. 1A, chloramphenicol acetyltransferase (CAT) activity directed by p(pGBS)$_4$E1bCAT was positively regulated by GCMa. Moreover, this GCMa-mediated transcriptional activation was further stimulated about four fold in the presence of forskolin (Fig. 1A). To investigate the signaling pathway activated by forskolin, several kinase inhibitors, including H89, PD 98059, and SB 203580, were tested in transient-expression experiments for blockage of the stimulatory effect of forskolin. As shown in Fig. 1A, the stimulatory effect of forskolin was significantly inhibited by H89, a specific inhibitor of the cAMP-dependent PKA, but not by the MEK inhibitor, PD 98059, nor by the p38 mitogen-activated protein kinase inhibitor, SB 203580.

Since forskolin stimulates *syncytin* gene expression and cell-cell fusion in placental BeWo cells, we tested whether forskolin also stimulates endogenous GCMa activity in BeWo cells. To this end, BeWo cells were transfected with p(pGBS)$_4$E1bLUC or p(Mut)$_5$E1bLUC, which contains a mutant pGBS not recognized by GCMa, and treated with or without forskolin. The luciferase activity directed by p(pGBS)$_4$E1bLUC was significantly higher than that shown by p(Mut)$_5$E1bLUC in the absence of forskolin, a finding suggestive of a specific response of p(pGBS)$_4$E1bLUC to the endogenous GCMa proteins (Fig. 1B). Moreover, the luciferase activity directed by p(pGBS)$_4$ E1bLUC, but not p(Mut)$_5$E1bLUC, was significantly stimulated by forskolin (Fig. 1B). This forskolin-upregulated GCMa activity was counteracted by the addition of H89 or upon pPKI or pRevAB cotransfection (Fig. 1B). pPKI and pRevAB constructs encode a peptide inhibitor specific to PKAcata and a dominant-negative regulatory subunit of PKA, respectively. Therefore, these results suggested that PKA regulates GCMa activity in the forskolin-activated cAMP signaling pathway. This notion was also supported by the observation that the luciferase activity directed by p(pGBS)$_4$E1bLUC was significantly increased upon pPKAcata cotransfection in the absence of forskolin (Fig. 1B).

CBP is involved in the PKA-upregulated GCMa activity. Examination of the protein sequence of GCMa by PROSITE

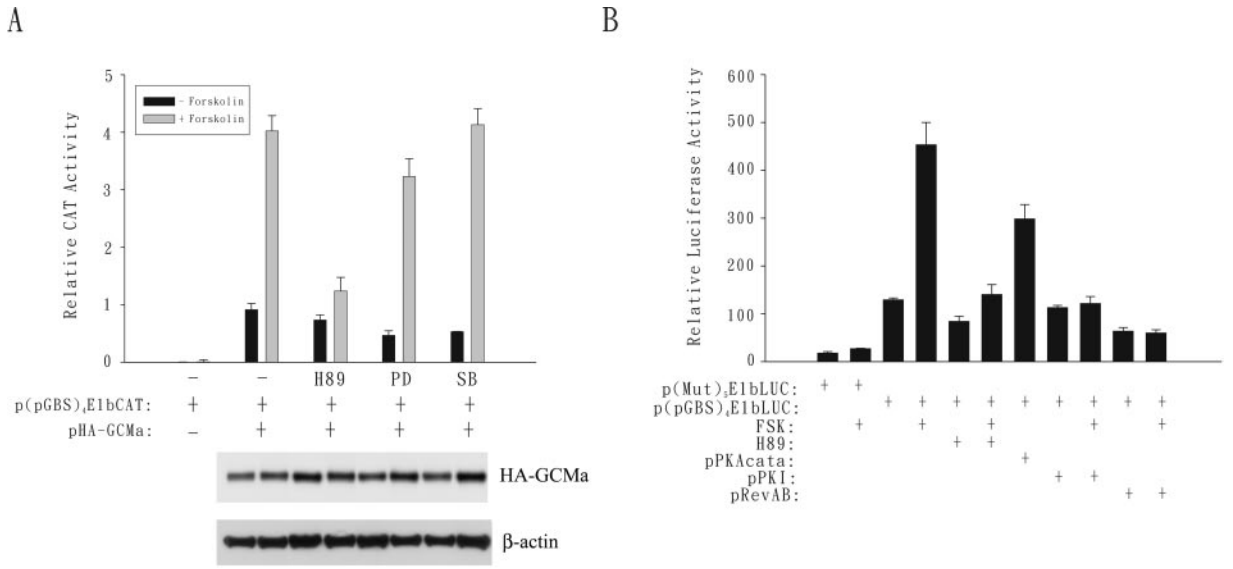

FIG. 1. Forskolin and PKA stimulate the transcriptional activity of GCMa. (A) 293T cells were transfected with 0.5 μg of $p(pGBS)_4E1bCAT$ alone or together with 0.5 μg of pHA-GCMa. At 24 h posttransfection, cells were mock-treated or treated with 50 μM forskolin or 50 μM forskolin plus 3 μM H89, 10 μM PD 98059, or 3 μM SB 203580 for another 24 h. Mean values and the standard errors of the mean (SEM) obtained from four independent transfection experiments are provided. The protein levels of HA-GCMa and β-actin (as a loading control) in each transfection group were detected by immunoblotting with HA-MAb and β-actin MAb, respectively. PD, PD 98059; SB, SB 203580. (B) BeWo cells were transfected with 0.5 μg of $p(pGBS)_4E1bLUC$ or $p(Mut)_5E1bLUC$ alone or together with 0.2 μg of pPKAcata, pPKI, or pRevAB. At 24 h posttransfection, cells were mock treated or treated with 50 μM forskolin or 50 μM forskolin plus 3 μM H89 for another 24 h. Mean values and the SEM obtained from three independent transfection experiments are provided. FSK, forskolin.

(25) and NetPhos (6) suggested several PKA consensus phosphorylation sites in GCMa. Indeed, GCMa was phosphorylated by the catalytic subunit of PKA in vitro (see Fig. S1A to D in the supplemental material). However, transient-expression experiments using mutant GCMa expression plasmids harboring mutations in these PKA phosphorylation sites did not demonstrate any adverse effect on the PKA-upregulated GCMa activity (see Fig. S1E in the supplemental material). Since CBP is an important downstream effector in the cAMP/PKA signaling pathway and functions as a coactivator for many transcription factors, we therefore tested whether CBP was involved in the regulation of GCMa activity by PKA. 293T cells were transfected with $p(pGBS)_4E1bLUC$, pHA-GCMa, and plus increasing amounts of pCBP-HA. As shown in Fig. 2A, CBP enhanced the transcriptional activity of GCMa in a dose-dependent manner. Interestingly, this positive effect of CBP was further increased when pPKAcata was cotransfected (Fig. 2A). We also tested the effect of a CBP siRNA on the forskolin-upregulated GCMa activity by cotransfecting 293T cells with $p(pGBS)_4E1bCAT$, pHA-GCMa, and CBP siRNA. As shown in Fig. 2B (left panel), the CBP siRNA, but not the unrelated GL2 siRNA, efficiently knocked down the endogenous CBP transcript in 293T cells based on RT-PCR (Fig. 2B, left panel). Correspondingly, CBP siRNA, but not GL2 siRNA reduced GCMa-mediated transcriptional activation in the presence or absence of forskolin (Fig. 2B, right panel), suggesting that CBP is an important regulator for GCMa-mediated transcriptional activation.

We further tested whether GCMa physically interacts with CBP by transfecting 293T cells with pHA-GCMa and pCBP-HA, followed by immunoprecipitation with GCMa antibody and immunoblotting with HA MAb. As shown in Fig. 2C (left panel), GCMa specifically interacted with CBP. Moreover, this interaction was stimulated when pPKAcata was cotransfected (Fig. 2C, left panel). To investigate the role of PKA in regulating the interaction between GCMa and CBP, we tested whether dephosphorylation of GCMa and CBP by λ-PPase affects the interaction between the two proteins. 293T cells were cotransfected with pHA-GCMa and pPKAcata or with pCBP-HA and pPKAcata, and the HA-GCMa and CBP-HA proteins were immunoprecipitated, respectively. The immune complexes were treated with λ-PPase or λ-PPase plus Na_3VO_4, a phosphatase inhibitor. The treated HA-GCMa and CBP-HA immune complexes were incubated with recombinant Flag-CBP and GCMa-Flag proteins, respectively, in pull-down assays. As shown in Fig. 2C (right panel), the interaction between GCMa and CBP was decreased when the precipitated HA-GCMa, but not CBP-HA, was pretreated with λ-PPase. However, this interaction between HA-GCMa and Flag-CBP was not affected when λ-PPase was inhibited by Na_3VO_4. These results suggested that PKA may modify the phosphorylation status of GCMa thereby increasing its CBP-binding activity.

To study the interaction between GCMa and CBP in placental cells, previously established stable BeWo cells expressing HA-GCMa (43) were used for coimmunoprecipitation analyses. Interaction between HA-GCMa and endogenous CBP was barely detectable in the stable BeWo cells (data not shown). However, when the stable BeWo cells were treated with forskolin, specific interaction between HA-GCMa and endogenous CBP was detected by immunoprecipitation and immunoblotting with HA MAb and CBP antibodies from different sources (Fig. 3A). As a control, this interaction was not

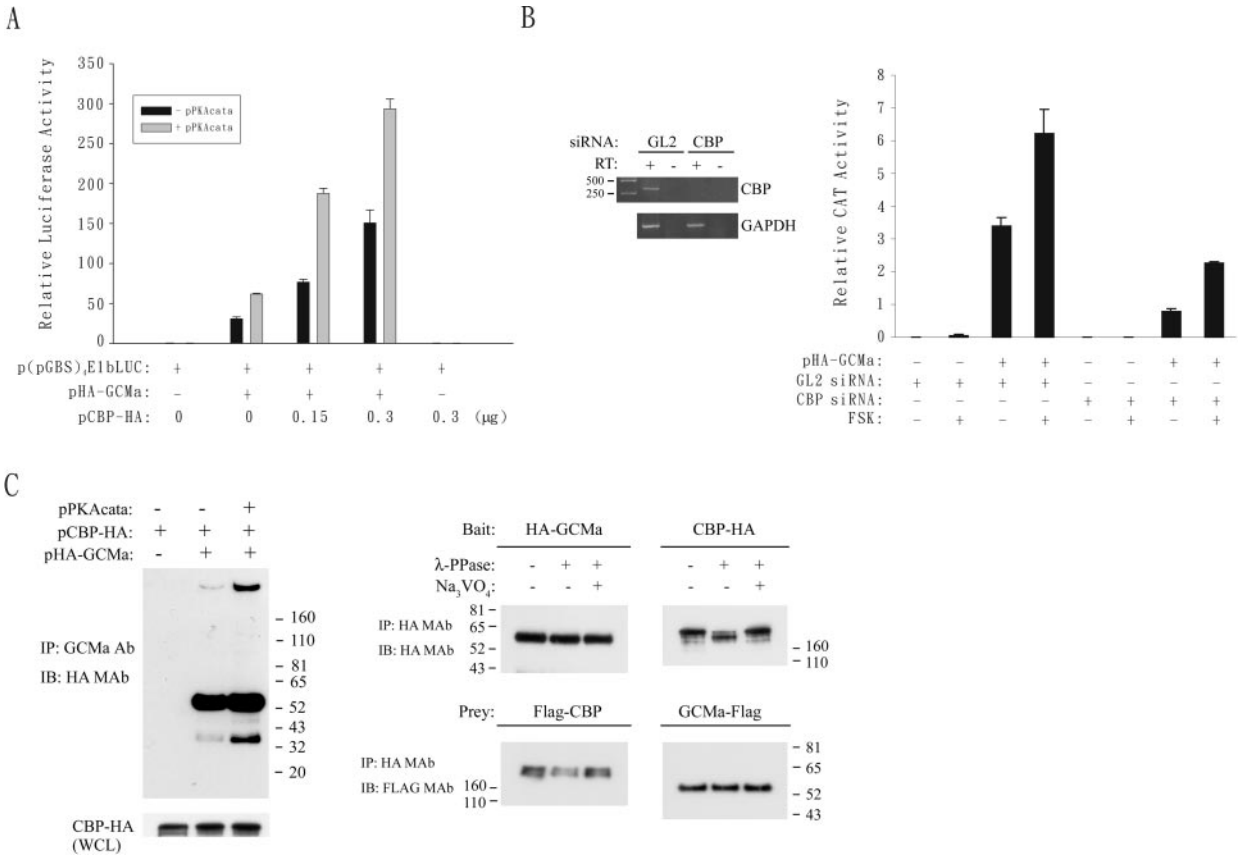

FIG. 2. Functional and physical interaction of GCMa and CBP. (A) CBP and PKA synergistically enhance GCMa-mediated transcriptional activation. 293T cells were transfected with 0.3 μg of $p(pGBS)_4E1bLUC$ alone or together with 0.3 μg of pHA-GCMa or 0.3 μg of pHA-GCMa plus the indicated amount of pCBP-HA (■). In a separate set of experiments, 0.1 μg of pPKAcata was included in each transfection group (▨). Mean values and SEM obtained from four independent transfection experiments are provided. (B) Inhibition of GCMa-mediated transcriptional activation by CBP siRNA. 293T cells were transfected with the indicated combinations of 0.3 μg of $p(pGBS)_4E1bCAT$, 0.3 μg of pHA-GCMa, and 10 nM GL2 or CBP siRNA. At 24 h posttransfection, cells were mock-treated or treated with 30 μM forskolin for another 24 h. Mean values and the SEM obtained from three independent transfection experiments are provided. The efficacy of CBP siRNA was analyzed by RT-PCR of 293T cells transfected with 10 nM GL2 or CBP siRNA. (C) GCMa interacts with CBP. 293T cells were transfecetd with 1 μg of pCBP-HA alone or together with 1 μg of pHA-GCMa or 1 μg of pHA-GCMa plus 0.1 μg of pPKAcata. At 48 h posttransfection, cells were harvested for coimmunoprecipitation assays as described in Materials and Methods. The protein level of CBP-HA in the whole-cell lysate (WCL) is presented. Of note, the interaction of GCMa and CBP was enhanced in the presence of PKAcata. IP, immunoprecipitation; IB, immunoblot. (D) PKA regulates the CBP-binding activity of GCMa. 293T cells were transfected with 5 μg of pHA-GCMa or pCBP-HA. At 48 h posttransfection, cells were harvested and immunoprecipitated with HA-MAb. The immune complexes were mock treated or treated with 200 U of λ-PPase plus or minus 20 mM Na_3VO_4. The treated HA-GCMa and CBP-HA complexes were then incubated with 1 μg of recombinant Flag-CBP and GCMa-Flag proteins, respectively, for pull-down analysis as described in Materials and Methods.

detected using an unrelated antibody against the GAL4 DNA-binding domain, Gal4 antibody, for immunoprecipitation (Fig. 3A).

We further investigated whether forskolin could stimulate the occupancy of syncytin promoter by GCMa and CBP in BeWo cells by ChIP assays. As expected, the level of syncytin transcript was significantly increased in BeWo cells treated with forskolin in Northern analysis (Fig. 3B, lower panel). Under ChIP analysis, occupancy of GCMa and CBP on pGBS in the syncytin promoter was significantly increased in forskolin-treated BeWo cells compared to the untreated BeWo cells (Fig. 3B, upper panel). To test the effect of GCMa and CBP on *syncytin* promoter activity, we performed transient-expression experiments in JAR cells, in which *syncytin* gene expression has been shown to be stimulated by forskolin (35). JAR cells were transfected with pHA-GCMa, pCBP-HA, and pLUC(25468-30953), a reporter construct of *syncytin* promoter with two functional GBSs. As shown in Fig. 3C, the luciferase activity directed by pLUC(25468-30953) was positively stimulated by GCMa or CBP. Moreover, the luciferase activity was further stimulated when pHA-GCMa and pCBP-HA were cotransfected (Fig. 3C). Taken together, these results suggested that the cAMP/PKA signaling pathway activated by forskolin leads to an increased association of GCMa and CBP with the syncytin promoter and a concomitant increase in *syncytin* gene expression.

Mapping of the interacting domains of GCMa and CBP. We next characterized the interaction between GCMa and CBP by mapping their interacting domains. To map the CBP-interacting domain(s) of GCMa, GST pull-down assays were performed by incubating a series of GST-GCMa fusion proteins with cell lysate of 293T cells transfected with pCBP-HA, fol-

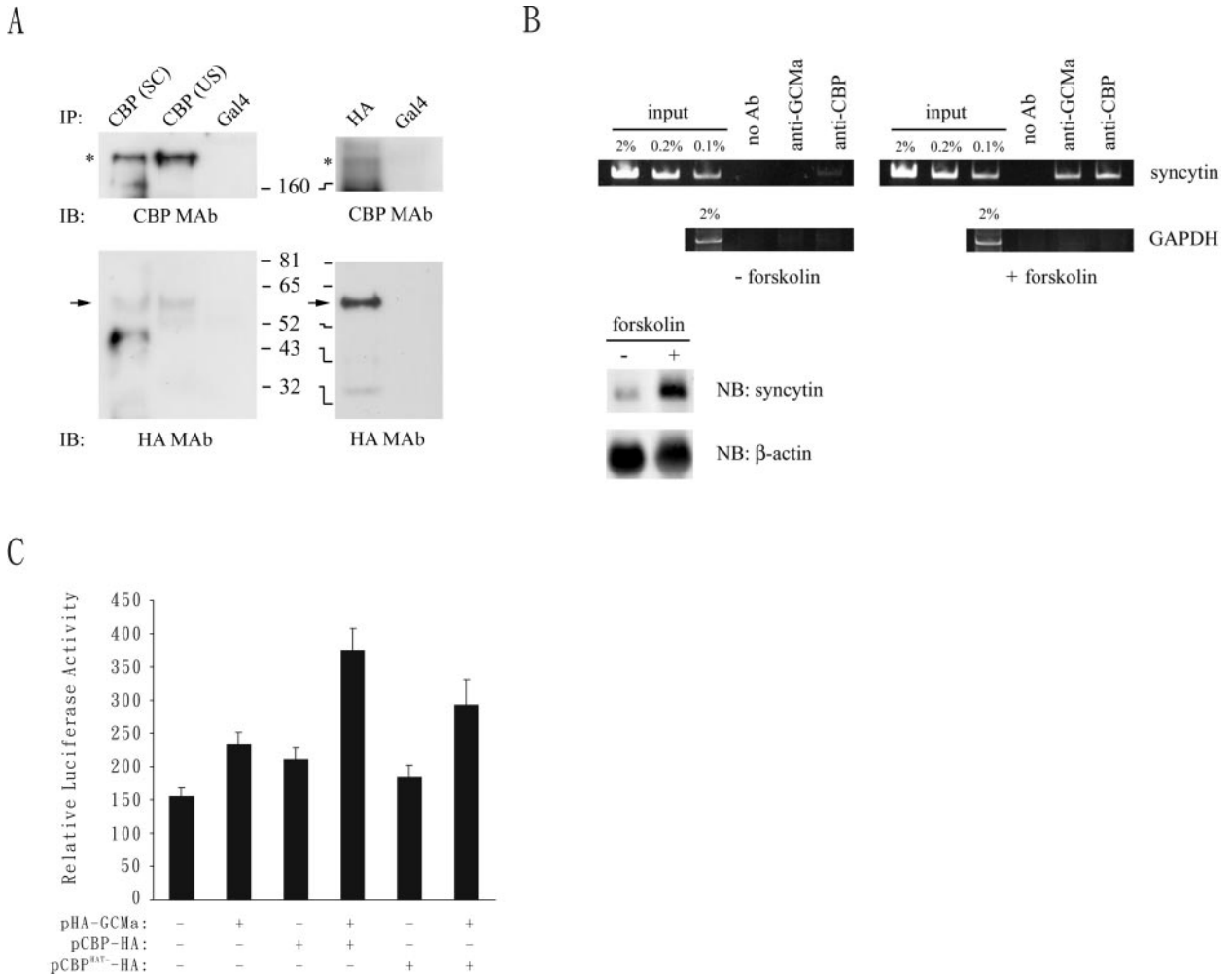

FIG. 3. Regulation of syncytin promoter by GCMa and CBP. (A) Physical interaction between GCMa and CBP in placental cells. Stable BeWo cells expressing HA-GCMa were treated with 30 μM forskolin for 24 h and then analyzed for the interaction between HA-GCMa and endogenous CBP by coimmunoprecipitation assays with the indicated combinations of antibodies. Asterisk and arrow indicate the CBP and the HA-GCMa protein, respectively. SC, Santa Cruz; US, Upstate. (B) Association of GCMa and CBP with the syncytin promoter is stimulated by forskolin. BeWo cells were treated with or without 50 μM forskolin for 12 h and analyzed by ChIP assays for a promoter region covering pGBS in the *syncytin* gene or for a promoter region in the *GAPDH* gene. A reaction was performed in the absence of antibody as a control (no Ab). Serial dilutions of input chromatin DNA were analyzed in PCRs with primers for the *syncytin* promoter. Mock- or forskolin-treated BeWo cells were also analyzed for the levels of syncytin and β-actin transcripts by Northern blotting (NB) using radioactive syncytin and β-actin cDNAs as probes. (C) Stimulation of *syncytin* promoter activity by GCMa and CBP. JAR cells were transfected with the indicated combinations of 0.1 μg of pLUC(25468-30953), 0.1 μg of pHA-GCMa, 0.1 μg of pCBP-HA, and 0.1 μg of $pCBP^{HAT-}$-HA. Mean values and the SEM obtained from five independent transfection experiments are provided.

lowed by immunoblotting with HA MAb. As shown in Fig. 4A, CBP interacted with two domains in GCMa, i.e., amino acids 1 to 220 and amino acids 349 to 436. The former essentially contains the GCM motif, whereas the later contains a C-terminal TAD. Likewise, to map the GCMa-interacting domain(s) of CBP, a series of GST-CBP fusion proteins were incubated with HA-GCMa-containing cell lysate, followed by immunoblotting with HA MAb. As shown in Fig. 4B, two domains in CBP were identified for interaction with GCMa, i.e., amino acids 1 to 451 and amino acids 1460 to 1891. The former contains a region from the N terminus to the C/H1 domain, whereas the later contains a partial HAT domain and the C/H3 domain.

Acetylation of GCMa by CBP does not affect its DNA-binding activity. Since CBP interacts with GCMa and has an intrinsic HAT activity that acetylates nonhistone proteins, we now tested whether CBP could acetylate GCMa. In vitro acetylation reactions were performed by incubating purified recombinant Flag-CBP and MBP or MBP-GCMa proteins in the presence or absence of Ac-CoA, followed by immunoblotting with Ac-K MAb. As shown in Fig. 5A, CBP specifically acetylated MBP-GCMa only in the presence of Ac-CoA. Similar reactions with MBP as the substrate did not reveal any acetylation signals. We further tested whether GCMa acetylation in the stable HA-GCMa-expressing BeWo cells could be regulated by forskolin. As shown in Fig. 5B, acetylation of HA-

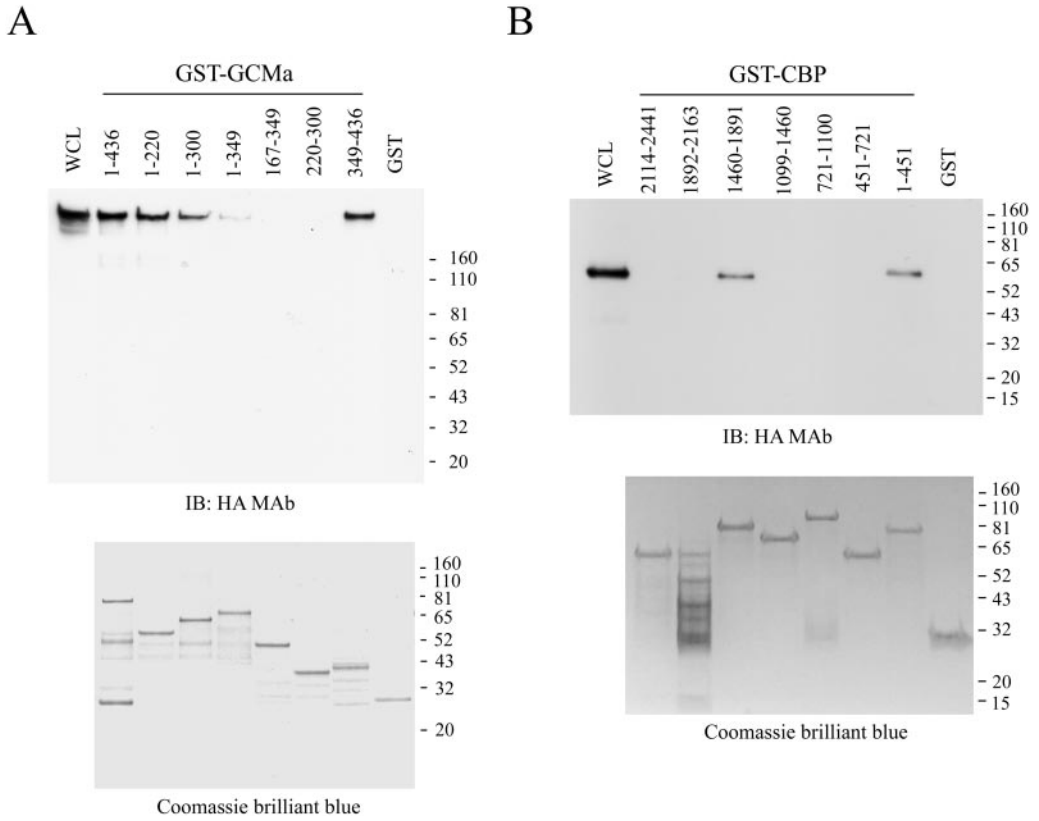

FIG. 4. Mapping of the interacting domains of GCMa and CBP. (A) CBP interacts with the GCM motif and the C-terminal TAD of GCMa. (B) GCMa interacts with a region from the N terminus to the C/H1 domain of CBP, as well as a partial HAT domain and the C/H3 domain of CBP. 293T cells were transfected with 5 μg of pCBP-HA and 2 μg of pHA-GCMa, respectively. At 48 h posttransfection, cells were harvested, and 200 μg of WCL was incubated with 2.5 μg of the indicated GST fusion protein for GST pull-down assays as described in Materials and Methods. Western analyses of 1/20 of whole-cell lysate (WCL) in panel A and 1/50 of WCL in panel B were performed. The lower panels are Coomassie brilliant blue stainings of GST fusion proteins used in pull-down assays.

GCMa was detected in the stable BeWo cells treated with forskolin, which was further enhanced in cells treated with forskolin and TSA, a histone deacetylase inhibitor. To test whether acetylation of GCMa affects its DNA-binding activity, unacetylated or acetylated MBP-GCMa was incubated with a radiolabeled pGBS probe in an EMSA. We first performed in vitro acetylation reactions with 1.5 μg of MBP-GCMa and increasing amounts (100, 200, 400, and 800 ng) of CBP in the presence or absence of Ac-CoA. The level of acetylated MBP-GCMa increased with increasing amounts of CBP and reached a plateau at 400 ng of CBP in the presence of Ac-CoA (Fig. 5C). A portion of each reaction was used for band-shift reactions to compare the DNA-binding activity of acetylated and unacetylated MBP-GCMa. As shown in Fig. 5C, although the fraction of acetylated MBP-GCMa was gradually increased in the total amount of MBP-GCMa used in the band-shift reactions, we did not observe a significant difference in the formation of DNA-protein complex for acetylated MBP-GCMa compared to unacetylated MBP-GCMa. Taken together, these results suggested that GCMa is an acetylation substrate of CBP. GCMa acetylation by CBP does not significantly affect the DNA-binding activity of GCMa.

CBP-mediated acetylation of GCMa increases the protein stability of GCMa. We further examined whether CBP could acetylate GCMa in vivo. Since GCMa has a similar mobility to the heavy chain of immunoglobulin G, which may impede acetylation analysis with Ac-K MAb, we therefore constructed the Gal4-GCMa fusion expression construct, pGal4-GCMa-Flag. 293T cells were cotransfected with different combinations of pGal4-GCMa-Flag, pCBP-HA, pCBP^{HAT-}-HA, and pPKAcata. As shown in Fig. 6A, acetylation of Gal4-GCMa-Flag was detected when pCBP-HA was cotransfected. Moreover, this acetylation was further enhanced in the presence of PKAcata (Fig. 6A, compare lanes 3 and 4). The observed acetylation depended on the HAT activity of CBP because the HAT-null mutant CBP^{HAT-}-HA failed to acetylate GCMa (Fig. 6A, lane 5). Interestingly, we also observed an increased protein level of Gal4-GCMa-Flag in 293T cells cotransfected with pGal4-GCMa-Flag, pCBP-HA, and pPKAcata (Fig. 6A, lane 4). This was unlikely to be due to differential transfection efficiencies because the level of the internal control protein, HA-EGFP, was similar in each transfection group. Therefore, we speculated that acetylation of GCMa by CBP increases the protein stability of GCMa. To test this hypothesis, pulse-chase experiments were performed in 293T cells transfected with pHA-GCMa alone, pHA-GCMa and pCBP-Flag, or pHA-GCMa and pCBP-Flag plus pPKAcata. As shown in Fig. 6B, HA-GCMa maintained a half-life of ca. 90 min, which was

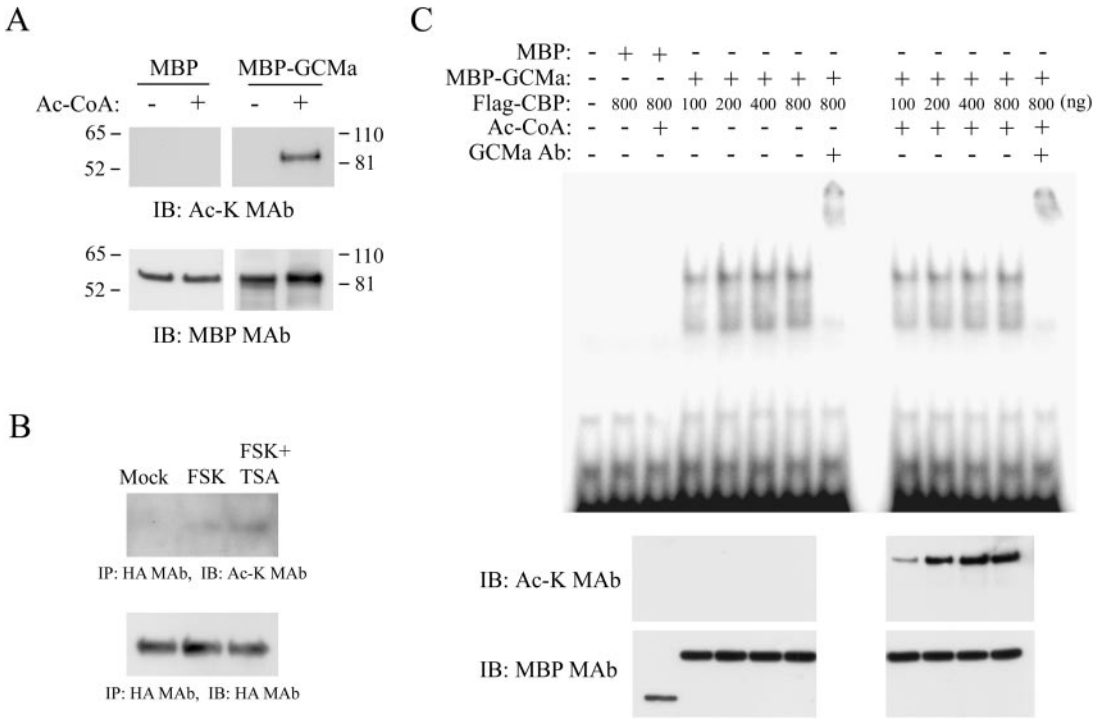

FIG. 5. In vitro and in vivo acetylation of GCMa. (A) In vitro acetylation of GCMa by CBP. 1.5 μg of MBP or MBP-GCMa fusion protein was incubated with 150 ng of recombinant Flag-CBP protein in a reaction mixture with or without Ac-CoA. The reaction mixtures were analyzed by immunoblotting with Ac-K MAb and MBP MAb, respectively. (B) In vivo acetylation of GCMa in placental cells. Stable BeWo cells expressing HA-GCMa were mock treated or treated with 10 μM forskolin alone or together with 50 ng of TSA/ml for 24 h. Cells were then harvested for the acetylation analysis of HA-GCMa as described in Materials and Methods. (C) Acetylation of GCMa does not significantly affect the DNA-binding activity of GCMa. A total of 1.5 μg of MBP or MBP-GCMa fusion protein was incubated with the indicated amount of recombinant Flag-CBP protein in a reaction mixture with or without Ac-CoA. One-thirtieth of each reaction mixture was analyzed by EMSA with a ^{32}P-labeled pGBS oligonucleotide. The complex of MBP-GCMa and pGBS was further verified by supershift reactions with GCMa antibody. For comparison, the levels of unacetylated and acetylated MBP-GCMa used for EMSA were analyzed by immunoblotting with MBP MAb and Ac-K MAb.

further prolonged in the presence of Flag-CBP or Flag-CBP plus PKAcata.

Recently, we demonstrated that GCMa can be ubiquitinated and degraded by the 26S proteasome (43). Since both ubiquitination and acetylation occur in the ε-amino group of lysine residues in substrate proteins, we now tested whether ubiquitination of GCMa could be counteracted upon GCMa acetylation by CBP. 293T cells were cotransfected with different combinations of pGCMa-Myc, pHA-Ub, pCBP-Flag, and pPKAcata. The level of ubiquitinated GCMa-Myc was analyzed by immunoprecipitation with Myc MAb and immunoblotting with HA MAb. As shown in Fig. 6C, the level of ubiquitinated GCMa-Myc was decreased in the presence of pPKAcata and was further decreased in the presence of CBP or CBP plus PKAcata. Taken together, these results suggest that CBP-mediated acetylation of GCMa prevents ubiquitination of GCMa and thereby increases the protein stability of GCMa.

Identification of CBP acetylation sites in GCMa. Inspection of GCMa protein sequence revealed 28 lysine residues as potential acetylation sites for CBP. Therefore, we now attempted to identify the CBP acetylation sites by first characterizing the domains in GCMa acetylated by CBP. We performed in vitro acetylation assays with recombinant Flag-CBP and a series of GST-GCMa fusion proteins. As shown in Fig. 7 (left panel), CBP-mediated acetylation was detected in most domains (amino acids 1 to 220, 1 to 300, 300 to 436, and 349 to 436) covering the whole GCMa polypeptide except the domain of amino acids 167 to 349. Therefore, the CBP acetylation sites in GCMa were localized to its N-terminal domain of amino acids 1 to 167 and C-terminal domain of amino acids 349 to 436, which also well correlated with the CBP-interacting domains in GCMa.

In addition, we also performed acetylation analysis of GCMa in vivo by cotransfecting 293T cells with pCBP-HA, pPKAcata, and truncated pGal4-GCMa-Flag expression plasmids containing different regions of GCMa. As shown in Fig. 7A (right panel), among the three domains in the C terminus of GCMa (amino acids 300 to 349, 349 to 436, and 300 to 436) tested, two (amino acids 300 to 436 and amino acids 349 to 436) were found to be acetylated by CBP. Surprisingly, unlike the in vitro acetylation results, pGal4-GCMa-Flag expression plasmids encoding the N-terminal regions of GCMa were not acetylated by CBP. Based on these in vivo results, the CBP acetylation sites in GCMa were localized to its C-terminal domain of amino acids 349 to 436.

Since the GCMa N-terminal domain of amino acids 1 to 167 harbors a DNA-binding domain motif, but acetylation of recombinant GCMa proteins by CBP did not change the DNA-binding activity of GCMa (Fig. 5C), we concentrated on identifying CBP acetylation sites in the C terminus of GCMa, which

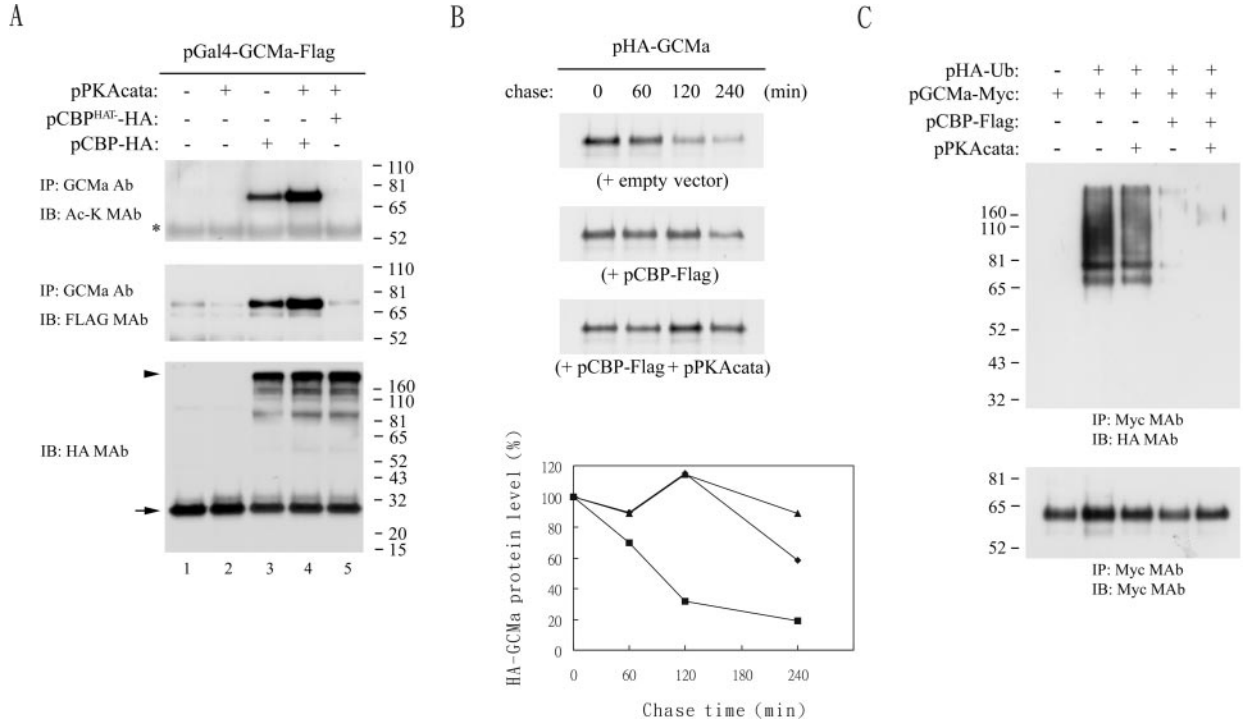

FIG. 6. CBP acetylates GCMa in vivo and increases GCMa protein stability. (A) 293T cells were transfected with 3 μg of pGal4-GCMa-Flag alone or together with the indicated combinations of 0.1 μg of pPKAcata, 3 μg of pCBP-HA, and 3 μg of pCBP^{HAT-}-HA. As an internal control of transfection efficiency, 0.3 μg of pHA-EGFP was also included in all transfection groups. At 48 h posttransfection, cells were harvested for acetylation analysis as described in Materials and Methods. The asterisk, arrow, and arrowhead indicate the heavy chain of IgG, the HA-EGFP protein, and the wild-type or mutant CBP-HA protein, respectively. Of note, CBP-HA further increased the level of acetylated Gal4-GCMa-Flag protein in the presence of PKAcata. (B) CBP-mediated acetylation increases the protein stability of GCMa. 293T cells were transfected with 3 μg of pHA-GCMa alone or together with 3 μg of pCBP-Flag or 3 μg of pCBP-Flag plus 0.1 μg of pPKAcata. At 36 h posttransfection, cells were analyzed by pulse-chase experiments as described in Materials and Methods. The lower panel shows the quantification of pulse-chase experiments by the bioimaging analyzer BAS-1500. Symbols: ■, pHA-GCMa plus empty vector; ◆, pHA-GCMa plus pCBP-Flag; ▲, pHA-GCMa plus pCBP-Flag plus pPKAcata. (C) Acetylation of GCMa by CBP prevents GCMa from ubiquitination. 293T cells were transfected with the indicated combinations of 7 μg of pGCMa-Myc, 7 μg of pHA-Ub, 3.5 μg of pCBP-Flag, and 1 μg of pPKAcata. At 24 h posttransfection, cells were treated with 40 μM MG132 for another 16 h, followed by immunoprecipitation with Myc MAb and immunoblotting with HA MAb or Myc MAb. The upper and lower panels indicate the levels of HA-ubiquitinated GCMa-Myc and total GCMa-Myc in the immune complexes, respectively.

also harbors a C-terminal TAD. Inspection of the protein sequence in amino acids 300 to 436 of GCMa revealed four potential lysine residues that could be acetylated, lysine^{349}, lysine^{367}, lysine^{406}, and lysine^{409}. To identify which of these lysine residues was actually acetylated by CBP, we constructed mutant pGal4-GCMa-Flag(300-436) expression plasmids harboring single or combined lysine to arginine mutations in the four lysine residues and tested their susceptibilities to CBP-mediated acetylation in 293T cells. As shown in Fig. 7B, compared to wild-type Gal4-GCMa-Flag(300-436), mutations at lysine^{367}, lysine^{406}, or lysine^{409} residues, but not lysine^{349}, resulted in reduced acetylation of mutant Gal4-GCMa-Flag(300-436) by CBP. Moreover, combined mutation of both lysine^{406} and lysine^{409} residues in Gal4-GCMa-Flag(300-436) (K2R) completely eliminated its acetylation by CBP (Fig. 7B). Similarly, combined mutation of lysine^{367}, lysine^{406}, and lysine^{409} residues (K3R) also eliminated its acetylation by CBP (data not shown). The observed differential acetylation of wild-type and mutant Gal4-GCMa-Flag(300-436) was not due to differential interactions of CBP with wild-type and mutant Gal4-GCMa-Flag(300-436) because a similar level of interaction was detected in coimmunoprecipitation experiments (data not shown). Taken together, these results suggested that of the three CBP acetylation sites (lysine^{367}, lysine^{406}, and lysine^{409}) in the C-terminal TAD of GCMa, lysine^{406} and lysine^{409} are the primary ones.

CBP enhances the transcriptional activity of GCMa via its HAT and coactivator activities. We now tested whether CBP-mediated acetylation affects the activity of GCMa C-terminal TAD. CV1 cells, which have a very low level of endogenous CBP (47), were cotransfected with pG5LUC, pGal4-GCMa-Flag(300-436), and pCBP-HA or mutant pCBP^{HAT-}-HA. pG5LUC is a luciferase reporter plasmid containing five tandem copies of a GAL4 binding site. As shown in Fig. 8A (left panel), CBP significantly, whereas CBP^{HAT-} only marginally, increased the transcriptional activity of Gal4-GCMa-Flag(300-436). When the level of Gal4-GCMa-Flag(300-436) in each cotransfection group was analyzed, coexpression with CBP-HA, but not CBP^{HAT-}-HA, also increased the protein level of Gal4-GCMa-Flag(300-436) (Fig. 8A, right panel). Therefore, CBP-driven acetylation may stabilize Gal4-GCMa-Flag(300-436) and enhances the transcriptional activity of Gal4-GCMa-Flag(300-436). Correlatively, the enhancement effect of CBP

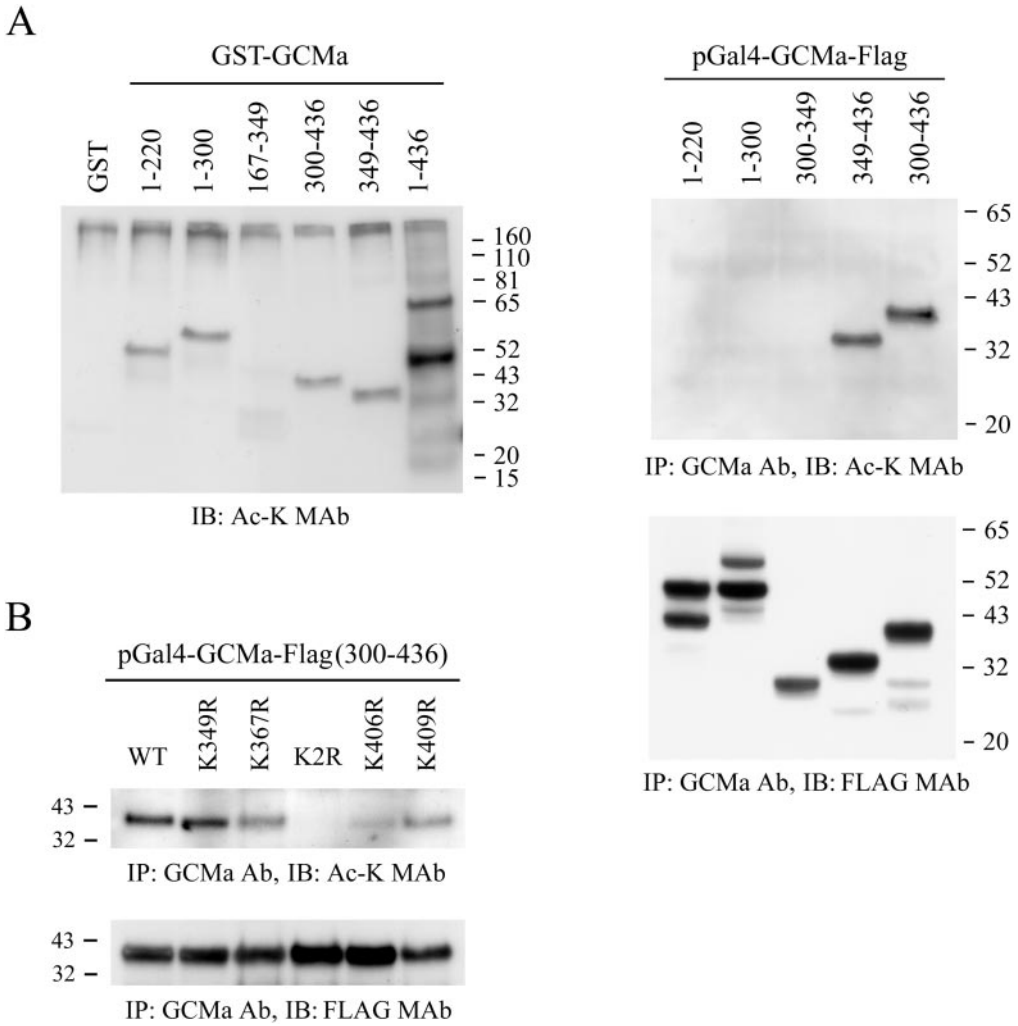

FIG. 7. Characterization of CBP-mediated GCMa acetylation sites. (A) Mapping of GCMa domains acetylated by CBP. To map the acetylation domains in GCMa in vitro (left panel), 0.2 μg of GST or the indicated GST-GCMa fusion protein was incubated with 0.4 μg of Flag-CBP and analyzed by in vitro acetylation reactions. To map the acetylation domains in GCMa in vivo (right panel), 293T cells were transfected with 0.5 μg of pCBP-HA, 0.1 μg of pPKAcata, and 2 μg of the indicated pGal4-GCMa-Flag expression plasmids for truncated GCMa proteins. At 48 h posttransfection, cells were harvested for acetylation analysis as described in Materials and Methods. The levels of immunoprecipitated Gal4-GCMa-Flag truncated proteins are given (lower right panel). (B) Mapping of CBP acetylation sites in the GCMa C-terminal TAD. 293T cells were transfected with 0.5 μg of pCBP-HA, 0.1 μg of pPKAcata, and 2 μg of the indicated wild-type or mutant pGal4-GCMa-Flag(300-436) expression plasmid. At 48 h posttransfection, cells were harvested for acetylation analysis as described in Materials and Methods. The levels of immunoprecipitated wild-type and mutant Gal4-GCMa-Flag(300-436) proteins are shown in the lower panel.

on GCMa-upregulated *syncytin* promoter activity was also stronger than that of CBP^{HAT-} in JAR cells (Fig. 3C).

We further tested whether CBP-mediated acetylation of lysine^{367}, lysine^{406}, and lysine^{409} in GCMa affects its TAD activity. CV1 cells were transfected with different combinations of pG5LUC, pGal4-GCMa-Flag(300-436), K2R, K3R, and pCBP-HA. Both K2R and K3R had a lower transcriptional activity than Gal4-GCMa-Flag(300-436) in the absence CBP (Fig. 8B, left panel). When cotransfected with pCBP-HA, CBP enhanced the transcriptional activity of Gal4-GCMa-Flag(300-436), K2R, and K3R, with the highest response being that of Gal4-GCMa-Flag(300-436) (Fig. 8B, left panel). Since CBP was unlikely to have acetylated K2R and K3R and CBP did not increase the protein levels of K2R and K3R as it did for the wild type (Fig. 8B, right panel), the mutated lysine residues may play important roles in maintaining the transcriptional activity and the protein stability of GCMa C-terminal TAD. Moreover, CBP's enhancement of K2R- and K3R-mediated transcriptional activation was very likely due to the coactivator activity of CBP. Taken together, these results suggested that lysine^{367}, lysine^{406}, and lysine^{409} in the GCMa C-terminal TAD modulate its transcriptional activity and that CBP-mediated acetylation of these residues increases its protein stability with a concomitant increase in transcriptional activation.

GCMa ubiquitination is regulated by CBP-mediated acetylation of the lysine^{367}, lysine^{406}, and lysine^{409} residues in GCMa. We next investigated whether acetylation of lysine^{367}, lysine^{406}, and lysine^{409} by CBP has any effect on GCMa ubiquitination. We first assayed CBP-mediated acetylation of full-length wild-type and lysine-to-arginine mutant GCMa by trans-

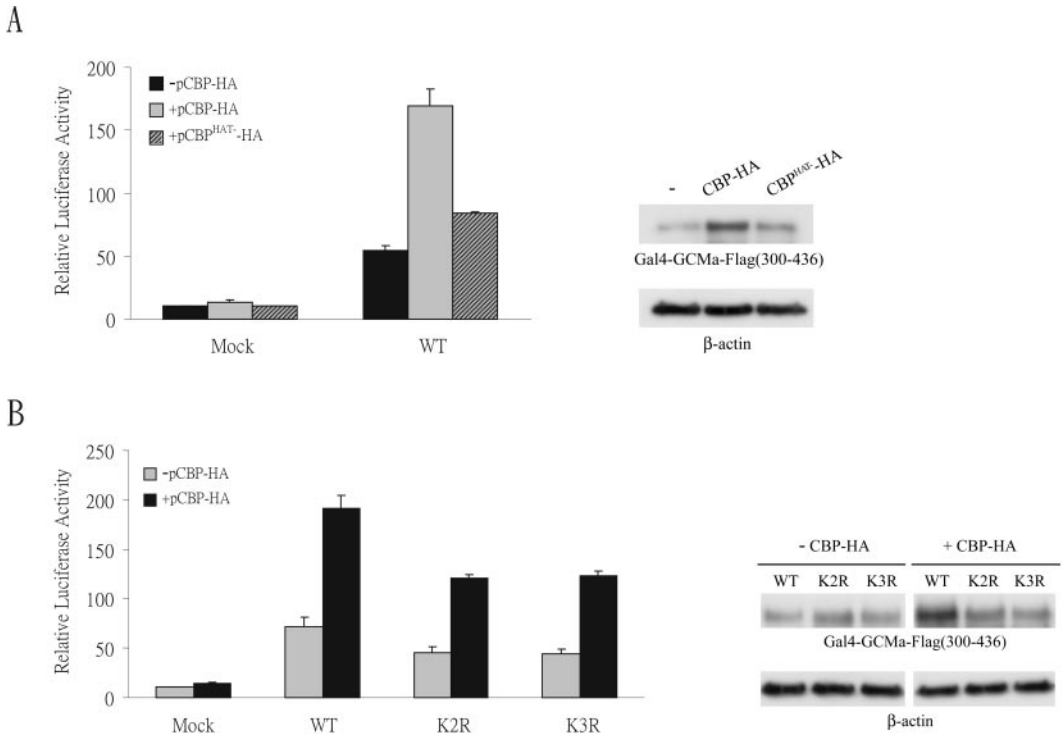

FIG. 8. CBP-mediated acetylation enhances GCMa-mediated transcriptional activation by increasing its protein stability. (A) Acetylation of GCMa TAD by CBP increases its transcriptional activity and protein stability. CV1 cells were cotransfected with 0.5 μg of pG5LUC, 0.5 μg of pGal4-GCMa-Flag(300-436), and 0.5 μg of pCBP-HA or mutant pCBP^{HAT-}-HA. Mean values and the SEM obtained from three independent transfection experiments are provided. The protein level of Gal4-GCMa-Flag(300-436) was analyzed by immunoblotting with FLAG MAb (right panel). (B) Roles of lysine^{367}, lysine^{406}, and lysine^{409} in the CBP-enhanced transcriptional activity of GCMa C-terminal TAD. CV1 cells were transfected with different combinations of 0.5 μg of pGal4-GCMa-Flag(300-436), pGal4-GCMa-Flag(300-436)K2R, pGal4-GCMa-Flag(300-436)K3R, and pCBP-HA. Mean values and the SEM obtained from three independent transfection experiments are listed. The protein level of Gal4-GCMa-Flag(300-436) and its mutants was analyzed by immunoblotting with FLAG MAb (right panel).

fecting 293T cells with pCBP-Flag and wild-type pGCMa-Myc or mutant pGCMa-Myc-K349R, -K2R, and -K3R. As shown in Fig. 9A, acetylation of wild-type GCMa-Myc was specifically detected when CBP-Flag, but not CBP^{HAT-}-Flag, was coexpressed, suggesting that this acetylation was dependent on the HAT activity of CBP. Acetylation of wild-type GCMa-Myc and mutant GCMa-Myc-K349R by CBP was detected to a similar level, whereas mutants GCMa-Myc-K2R and -K3R were only weakly acetylated by CBP (Fig. 9A). The lysine-to-arginine mutation did not have an adverse effect on the interaction between CBP and the individual mutant GCMa-Myc compared to wild-type GCMa-Myc (Fig. 9A). These results suggested that lysine^{367}, lysine^{406}, and lysine^{409} are the major CBP acetylation sites in the full-length GCMa. Moreover, there are other lysine residues functioning as minor CBP acetylation sites because weak acetylation signals of GCMa-Myc-K2R and -K3R were still detected in the presence of CBP.

Subsequently, we tested the effect of acetylation of lysine^{367}, lysine^{406}, and lysine^{409} on GCMa ubiquitination. We compared ubiquitination of wild-type and lysine-to-arginine mutant GCMa by transfecting 293T cells with different combinations of pCBP-Flag, pHA-Ub, pGCMa-Myc, and pGCMa-Myc-K349R, -K2R, and -K3R. As shown in Fig. 9B, ubiquitination of wild-type and all mutant GCMa was detected to a similar level in the absence of CBP. Therefore, the three major acetylation sites and other lysine residues (possibly including the minor acetylation sites) are susceptible to ubiquitination when they are not acetylated by CBP. Interestingly, ubiquitination of wild-type GCMa-Myc and mutant GCMa-Myc-K349R, but not GCMa-Myc-K2R and -K3R, was counteracted in the presence of CBP (Fig. 9B). Therefore, when the major acetylation sites are not acetylated or are mutated into arginines (as in GCMa-Myc-K3R), other lysine residues (possibly including the minor acetylation sites) are more susceptible to ubiquitination than to CBP-mediated acetylation. These results suggested that acetylation of lysine^{367}, lysine^{406}, and lysine^{409} significantly protects GCMa from ubiquitination.

DISCUSSION

Trophoblastic fusion is essential for formation of the syncytiotrophoblast layer during placental development (5). It has long been known that fusion of cultured placental cells can be stimulated by forskolin (29, 40). However, the underlying mechanism of this phenomenon remained elusive until two recent observations shed new light on it. First, a placenta-specific fusogenic protein termed syncytin was identified and shown to mediate fusion of cultured placental cells (33). Second, forskolin was shown to stimulate expression of the *syncytin* gene in placental cells (33). These observations suggest that forskolin stimulates placental cell fusion via transcriptional regulation of *syncytin* gene expression.

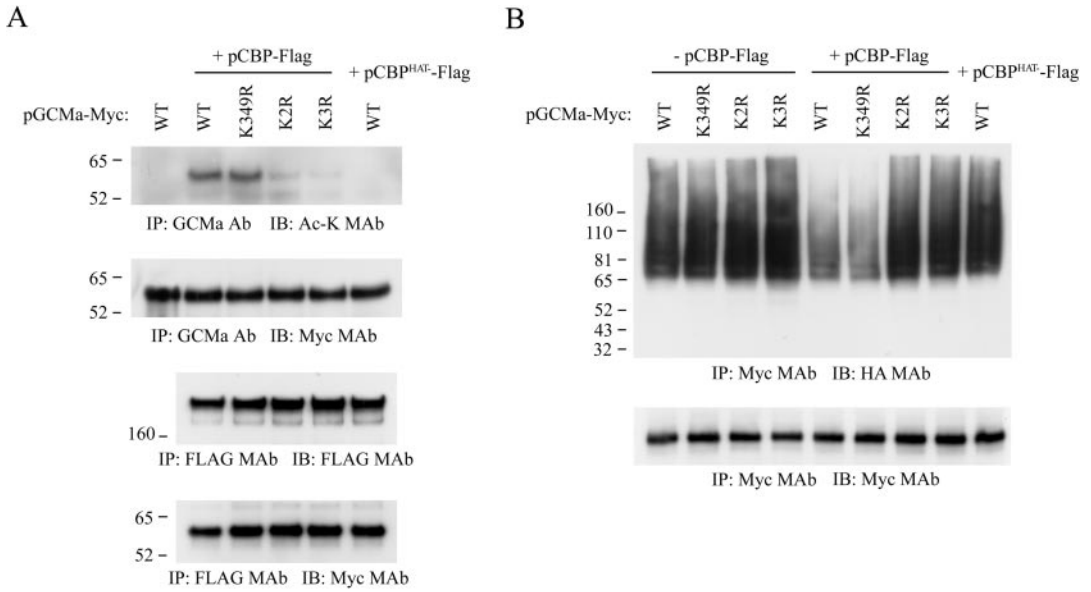

FIG. 9. GCMa ubiquitination is regulated by CBP-mediated acetylation. (A) Lysine^{367}, lysine^{406}, and lysine^{409} are the major CBP acetylation sites in GCMa. 293T cells were transfected with 4 μg of wild-type or mutant pGCMa-Myc and 1 μg of pCBP-Flag or pCBP^{HAT-}-Flag. At 48 h posttransfection, cells were harvested for acetylation analysis as described in Materials and Methods. In a separate set of experiments, cells were harvested for coimmunoprecipitation assays to verify similar levels of interaction between CBP-Flag and wild-type or mutant GCMa-Myc. (B) Acetylation of lysine^{367}, lysine^{406}, and lysine^{409} counteracts GCMa ubiquitination. Ubiquitination of wild-type and mutant GCMa-Myc in transfected 293T cells was analyzed as in Fig. 6C.

Earlier we had shown that the placental transcription factor, GCMa, regulates expression of *syncytin* gene (45). In the present study, we further confirmed that forskolin is able to stimulate GCMa-mediated transcriptional activation via PKA and CBP, two key components of the cAMP signaling pathway. Several lines of evidence support that PKA modulates the transcriptional activity of GCMa. First, forskolin was able to stimulate the transcriptional activity of GCMa in transient-expression experiments, while this effect was negated by the addition of H89, an inhibitor of cAMP-dependent PKA. Second, this forskolin-induced upregulation of GCMa transcriptional activity was also inhibited by the peptide inhibitor, PKI, and the dominant-negative regulatory subunit of PKA, RevAB, both of which specifically blocked the catalytic activity of PKA. Third, the transcriptional activity of endogenous and ectopic GCMa was enhanced in transient-expression experiments in the presence of the PKA catalytic subunit. Although we identified several major PKA phosphorylation sites in GCMa by in vitro kinase assays, serine-to-alanine mutagenesis of these sites did not have any adverse effect on PKA-upregulated GCMa transcriptional activity (see Fig. S1 in the supplemental material). Two possibilities may explain this observation. First, cryptic PKA phosphorylation sites may exist in GCMa that were not identified by our in vitro assay, and these may play important roles in regulation of GCMa activity. A more sensitive analytical tool such as mass spectrometry could characterize these cryptic PKA phosphorylation sites in future investigations. Second, PKA may indirectly modulate GCMa activity by cross-talking with other signaling pathways. Since the MEK inhibitor PD 98059 and the p38 mitogen-activated protein kinase inhibitor SB 203580 did not alter the effect of forskolin on GCMa activity (Fig. 1), the role of other signaling pathways on direct regulation of GCMa activity needs to be investigated.

In order to ascertain whether PKA phosphorylation of GCMa directly enhances GCMa transcriptional activity, we investigated the possibility that PKA may regulate the interaction between GCMa and other effectors in the cAMP/PKA signaling pathway. We demonstrated that CBP interacts with and acetylates GCMa and that PKA can positively regulate both events. In addition, PKA very likely modifies the phosphorylation status of GCMa to facilitate an interaction between GCMa and CBP because treatment of GCMa with λ-PPase decreases the interaction between GCMa and CBP. We are currently investigating how PKA modification of GCMa phosphorylation increases the CBP-binding activity of GCMa. Our results of this study are similar to what is known about the pituitary-specific transcription factor, Pit-1, which is a POU domain-containing protein. Pit-1 is required to mediate PKA-regulated expression of growth hormone (GH), prolactin, GH-releasing hormone receptor, and thyrotropin β-subunit genes (31, 46). Although Pit-1 is a direct target of PKA, mutation of the PKA phosphorylation sites in Pit-1 does not affect PKA-regulated Pit-1 activity (13, 41). Instead, PKA-regulated Pit-1 activity has been attributed to a physical interaction between CBP and Pit-1 (41, 46). Xu et al. (41) have further demonstrated that a C-terminal consensus PKA site in CBP is required for PKA to regulate Pit-1 activity. However, Zanger et al. (46) have reported contradictory results showing that this consensus PKA site in CBP does not play any significant role in PKA-regulated Pit-1 activity. Nevertheless, it is a common mechanism by which cAMP/PKA signaling pathway regulates GCMa and Pit-1 activity via a direct interaction between CBP and GCMa or Pit-1.

Protein acetylation regulates the biological activities of histone and nonhistone proteins. Acetylation occurs on all core histones (H3, H4, H2A, and H2B) at the evolutionarily conserved lysine residues located at the N terminus and consequently changes chromatin architecture thereby increasing transcriptional activity. In general, acetylation of transcription factors can alter their activities at various levels, including DNA binding, transcriptional activity, their interactions with other proteins, nuclear transport, and protein turnover. A variety of nonhistone proteins have been demonstrated to be acetylated by CBP, including p53, c-Myc, and NF-κB, to name a few (9, 12). In the present study, we also identified GCMa as a bona fide CBP acetylation substrate. Acetylation of GCMa by CBP is stimulated by PKA, which is most likely due to the increased interaction between GCMa and CBP. Moreover, acetylation of GCMa by CBP increases the protein stability of GCMa by blocking GCMa ubiquitination. Although Pit-1 can interact with CBP, it is a poor CBP acetylation substrate and requires an additional factor to enhance its acetylation by CBP (34). Although CBP efficiently acetylates GCMa in vitro, the possibility of additional factors participating in CBP-mediated acetylation of GCMa in vivo cannot be ruled out since CBP can associate with other acetyltransferases, including P/CAF (44) and GCN5 (42).

In terms of transcriptional activation, we found that CBP enhances the transcriptional activity of GCMa C-terminal TAD via its HAT and coactivator activities. Several lines of evidence support this conclusion. First, the transcriptional activation mediated by Gal4-GCMa-Flag(300-436) was significantly enhanced by CBP, but not by CBP^{HAT-}, with a corresponding increase in protein level of Gal4-GCMa-Flag(300-436). Correlatively, the full enhancement effect of CBP on GCMa-regulated *syncytin* promoter activation requires the acetyltransferase activity of CBP. Second, acetylation of GCMa by CBP prevented GCMa from ubiquitination. Third, the CBP-enhanced transcriptional activity of K3R, which had the CBP acetylation sites (lysine^{367}, lysine^{406}, and lysine^{409}) mutated was still detected, although to a lesser degree. Therefore, stabilization of GCMa via the HAT activity of CBP, and connection between GCMa and the transcription machinery via the coactivator activity of CBP underlies the observed CBP-enhanced transcriptional activity of GCMa. Unfortunately, whether CBP-mediated acetylation of the C-terminal TAD of GCMa upregulates its transcriptional activity is complicated by the acetylation-dependent protein stabilization of TAD. An in vitro transcription assay for acetylated and unacetylated GCMa may help to resolve this issue.

Although GCMa contains 28 lysine residues as potential acetylation and ubiquitination sites, lysine^{367}, lysine^{406}, and lysine^{409} were identified as the major CBP acetylation sites but not the major ubiquitination sites because changing these lysine residues into arginines in GCMa-Myc-K3R significantly reduced its level of acetylation mediated by CBP but not ubiquitination (Fig. 9A and B). Moreover, when the major acetylation sites are not acetylated or are mutated into arginines (as in GCMa-Myc-K3R), other lysine residues (possibly including the minor acetylation sites) are more susceptible to ubiquitination than to CBP-mediated acetylation. The ETS protein, ER81, is stabilized by p300-mediated acetylation at lysine^{33} and lysine^{116} (18). A conformational change in the acetylated ER81 or a shielding effect from acetylated ER81-associated factors has been proposed to prevent ER81 from ubiquitination. Whether similar mechanisms are applicable to the stabilization of acetylated GCMa is still an open question. However, considering the tremendously counteracting effect of CBP-mediated acetylation on ubiquitination of wild-type GCMa-Myc and mutant GCMa-Myc-K349R, it is feasible to speculate that acetylation of lysine^{367}, lysine^{406}, and lysine^{409} residues may either further promote acetylation or simply protect ubiquitination of other lysine residues (perhaps including the minor acetylation sites) in GCMa.

Previous studies have demonstrated a good correlation between increased cAMP synthesis and cell fusion in BeWo cells treated with forskolin (40). Moreover, addition of cAMP analogues to primary-culture trophoblast cells led to stimulation of syncytium formation and redistribution of PKA type IIα in syncytial cells (29). Recently, Frendo et al. (17) have demonstrated that suppression of syncytin expression by antisense oligonucleotides resulted in a decrease in fusion and differentiation of primary-culture trophoblast cells. These observations indicate that cAMP and PKA are upstream mediators and syncytin is a downstream effector involved in trophoblastic fusion. Since the interaction between syncytin and its receptors, ASCT1 and -2, can be a rate-limiting step in the fusion process, controlling expression of syncytin and its receptors by mediators provides a hierarchical regulation of trophoblastic fusion in placental development. Indeed, it is known that the level of ASCT1 mRNA was low and that forskolin can induce *syncytin*, but not *ASCT2*, gene expression in placental cells (35). Therefore, regulation of *syncytin* gene expression is an important step in forskolin-stimulated placental cell fusion. Together with the fact that GCMa is a key factor regulating *syncytin* gene expression, the importance of the forskolin-cAMP/PKA-GCMa-syncytin signal transduction pathway in controlling trophoblastic fusion during placental development is evident. Although the effect of the cAMP-PKA signaling pathway is multifaceted, and it is highly probable that additional mechanisms are involved in trophoblastic fusion induced by this pathway, based on the results of the present study, we propose a model for forskolin-stimulated placental cell fusion as follows. Treatment of placental cells with forskolin activates PKA, which in turn modifies the phosphorylation status of GCMa, thereby facilitating an interaction between GCMa and CBP. Concomitantly, the level of GCMa-CBP complex is increased and so does the level of stabilized acetylated GCMa protein, which further activates *syncytin* gene expression and promotes placental cell fusion.

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