Histone deacetylase 3 binds to and regulates the GCMα transcription factor

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Received December 5, 2005; Revised December 16, 2005; Accepted February 22, 2006

ABSTRACT

Human GCMα transcription factor regulates expression of syncytin, a placental fusogenic protein mediating trophoblastic fusion. Recently, we have demonstrated that CBP-mediated GCMα acetylation underlies the activated cAMP/PKA signaling pathway that stimulates trophoblastic fusion. Because protein acetylation is a reversible modification governed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), in this study we investigated the key HDACs responsible for deacetylation of GCMα and thus the reduction in GCMα activity to avoid unwanted fusion events that may have adverse effects on placental morphogenesis. We herein demonstrate that the HDAC inhibitor, trichostatin A (TSA), increases the level of acetylated GCMα and that HDAC1, 3, 4 and 5 interact with and deacetylate GCMα. Glutathione S-transferase (GST) pull-down assays further verified direct interaction between GCMα and HDAC3 or CBP and HDAC3. HDAC3 counteracts the transcriptional coactivator activity of CBP and the enhancement effect of CBP on GCMα-mediated transcriptional activation. Correlatively, we found in placental cells that HDAC3 associates with the proximal GCMα-binding site (pGBS) in the syncytin promoter and dissociates from pGBS in the presence of forskolin, which stimulates the association of CBP and GCMα with pGBS. Our studies support that trophoblastic fusion in placental morphogenesis depends on the regulation of GCMα activity by HAT and HDAC.

INTRODUCTION

The GCM (Glial Cell Missing) transcription factors, GCMα/1 and GCMβ/2, form a novel family of transcription factors with a characteristic zinc-containing DNA-binding domain (termed GCM motif) in their N-termini. GCM transcription factors play important roles in development. Drosophila GCM1 regulates the differentiation of glial cells from neuronal precursor cells, whereas murine GCMα and β regulate the formation of the placental syncytiotrophoblasts and parathyroid gland, respectively (1–6). Recently, zebrafish GCMb has been characterized and shown to be required for pharyngeal cartilage formation (7,8). Identification of target genes and associated factors of GCM transcription factors has shed new light on the molecular mechanisms underlying the above-mentioned developmental outcomes. For instance, Drosophila GCM1 regulates expression of repo (reverse polarity) and pnt (pointed) genes, which are two principal mediators of glial differentiation, whereas human GCMα regulates expression of syncytin gene, which encodes a placental fusogenic membrane protein facilitating trophoblastic fusion (9,10). Interestingly, Drosophila GCM1 can positively regulate its own expression via five GCM-binding sites in its promoter region (11). More recently, we have demonstrated that CBP directly interacts with and acetylates GCMα in the activated cAMP/PKA signaling pathway (12). In addition, Schubert et al. (13) have identified Pitx2, a paired-like homeodomain transcription factor, as a GCMα-interacting protein in a yeast two-hybrid screen. Synergistic transcriptional activation mediated by GCMα and Pitx2 has in fact been demonstrated in placental cells (13). In terms of protein turnover, GCMα can be targeted to the ubiquitin–proteasome degradation system by the F-box protein, FBW2 (14). Therefore, regulation of GCMα/1 activity during development can be achieved by post-translation modification, protein–protein interaction and autoregulation.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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Protein acetylation at the ε-amino group of lysine residue is a reversible and dynamic post-translational modification that regulates a variety of protein functions involved in chromatin assembly, protein–protein interaction, stability, DNA-binding activity, transcriptional activity and nuclear localization, to name but a few. The acetylation status of protein substrates is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of acetyl groups from acetyl-CoA to the ε-amino groups of lysine residues in proteins and play an important role in the regulation of transcriptional machinery and gene expression. For instance, CBP is a transcription coactivator connecting different transcription factors to the transcription machinery or providing a scaffold to form a multicomponent transcriptional regulatory complex (15). In addition, CBP has an intrinsic HAT activity to acetylate nucleosomal histones resulting in changes in chromatin structure and non-histone proteins affecting their biological activities (15). HDACs reverse the enzyme reaction catalyzed by HATs and promote transcriptional repression. Mammalian HDACs are categorized into three classes: class I RPD3-like HDACs (HDAC1, 2, 3 and 8) localize to the nucleus; class II HDAI-like HDACs (4, 5, 6, 7, 9 and 10) localize to both nucleus and cytoplasm; and class III SIR2-like HDACs (SIRT1-7) are structurally distinct from class I and II HDACs and require the cofactor NAD for their enzymatic activities (16).

Cell–cell fusion is essential for formation of the multinucleated syncytiotrophoblast layer during human placental development. Recently, we have demonstrated that GCMa is able to control placental cell fusion by upregulating syncytin gene expression (10). Moreover, we have further demonstrated that CBP is involved in the stimulation of GCMa activity via the cAMP/PKA signaling pathway (12). We have shown that CBP enhances GCMa-mediated transcriptional activation by acting as a transcriptional coactivator and as a HAT acetylating GCMa to prolong its protein stability (12). Therefore, the acetylation status of GCMa plays an important role in the regulation of GCMa activity. In this study we investigated the key HDACs that mediate deacetylation of GCMa. We demonstrated that HDAC1, 3, 4 and 5 are able to interact with and deacetylate GCMa. Moreover, we found that CBP specifically interacts with HDAC1 and 3. Because the interaction between CBP and HDAC3 is a new finding and may further complicate current understanding of how GCMa activity is regulated, we characterized the interaction between GCMa, HDAC3 and CBP and studied the regulation of GCMa-mediated transcriptional activation by HDAC3. Our study identified HDAC3 as a key factor reversing GCMa acetylation and suggests that HDAC3 can functionally attenuate the CBP-upregulated GCMa activity in the activated signaling pathway leading to placental cell fusion.

MATERIALS AND METHODS

Plasmid constructs

The expression plasmids pHA-GCMa, pPKAcata, pCBP-HA, pCBP-Flag, pCBP-HAT-Flag and pGal4-GCMa-Flag have been described previously (12). The pGCMa-Flag expression plasmid was constructed by cloning into the pEF1-MycHis (Invitrogen, Carlsbad, CA), a DNA fragment encoding human GCMa with a C-terminal triple FLAG tag. The HDAC expression plasmids pHDAC1-5, 2-, 3-, 4- and 5-Myc or -Flag were constructed by placing the respective ORF cDNA with a C-terminal four copies of Myc tag or a C-terminal one copy of FLAG tag under the control of cytomegalovirus (CMV) early promoter/enhancer. The reporter constructs, p(pGBS)E1bLUC and pLUC(25468–30953), have been described previously (12). All constructs were verified by DNA sequencing using the dyeode chain-termination method.

Cell culture, transfection and reporter gene assay

293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained at 37°C in HEPES-buffered DMEM supplemented with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). The human trophoblast cell lines, JEG3, BeWo and JAR, was obtained from ATCC and maintained at 37°C in F-12K medium supplemented with 15% FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml). For transient expression experiments, cells were transfected with the indicated amounts of reporter plasmid and expression plasmid as described in figure legends using the TransIT LT1 reagent (Mirus, Madison, WI). 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 described previously (12). Specific luciferase activities were normalized by protein concentration. Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL).

In vivo acetylation/deacetylation analysis

To study the effect of trichostatin A (TSA) on GCMa acetylation in vivo, 293T cells were transfected with different combinations of pGal4-Flag, pGal4-GCMa-Flag, pCBP-HA and pPKAcata. After 24 h post-transfection, cells were mock-treated or treated with the indicated amount of TSA for an additional 18 h. Cells were then harvested in the lysis buffer A containing 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween-20, 5% glycerol, 1 mM Na3VO4, 5 mM NaF and 1 mM phenylmethylsulfonyl fluoride (PMSF) for immunoprecipitation with a guinea pig anti-GCMa polyclonal antibody (GCMa Ab). The immune complexes were further analyzed by immunoblotting with a mouse anti-acetylated-lysine monoclonal antibody (Ac-K mAb, Cell Signaling, Beverly, MA). To identify the HDAC responsible for deacetylation of GCMa, 293T cells were transfected with different combinations of pGal4-Flag, pGal4-GCMa-Flag, pCBP-HA, pPKAcata and pHDAC1-5-Myc. After 48 h post-transfection, cells were harvested for acetylation analysis as described above.

Co-immunoprecipitation

To study the interaction between GCMa and HDAC, 293T cells were cotransfected with pHA-GCMa and pHDAC1-2-, 3-, 4- or 5-Myc as described in the legend of Figure 2A. After 48 h post-transfection, cells were harvested in
lysed with buffer A for immunoprecipitation with a mouse anti-HA monoclonal antibody (HA mAb, Sigma, St Louis, MO). The immune complexes were further analyzed by immunoblotting with the 9E10 Myc mAb (Roche, Mannheim, Germany). A reciprocal experiment was performed by immunoprecipitation of the cell lysate with Myc mAb, followed by immunoblotting with HA mAb. To study the interaction between CBP and HDAC3, 293T cells were cotransfected with pCBP-Flag and pHDAC1-, 2-, 3-, 4- or 5-Myc as described in the legend of Figure 3A. Co-immunoprecipitation was performed as in the aforementioned procedures. Characterization of the interaction between HDAC3, GCMa and CBP was performed by transfecting 293T cells with pHDAC3-Myc, pGCMa-Flag and pCBP-HA, followed by co-immunoprecipitation using the indicated combinations of HA and Myc mAbs and a mouse anti-FLAG monoclonal antibody (FLAG mAb, Sigma). To study the effect of HDAC3 on GCMa protein stability, 293T cells were transfected with different combinations of pHA-GCMa, pCBP-Flag and pHDAC3-Myc, followed by pulse-chase analysis as described previously (12).

**Sedimentation analysis**

293T cells were transfected with pCBP-Flag, pHA-GCMa and pHDAC3-Myc. After 48 h post-transfection, cells were harvested and the CBP–Flag complexes were immunoprecipitated using the above-mentioned procedures, followed by elution using 200 μg/ml of FLAG peptide. For sedimentation analysis, the eluted CBP–Flag complexes were analyzed in a 10 to 30% glycerol gradient in phosphate-buffered saline (PBS) in an SW40 Ti rotor (Beckman Coulter, Fullerton, CA) at 35,000 r.p.m. (150,000 × g) for 40 h. A total of 16 fractions were collected from the bottom of centrifuge tube and analyzed by immunoblotting using HA, FLAG and Myc mAbs, respectively.

**Glutathione S-transferase (GST) pull-down assay and interaction domain mapping**

The GST fusion protein expression vector pGEX6P-1 (Amersham Biosciences, Piscataway, NJ) was used for preparation of the GST–HDAC3 fusion protein in *Escherichia coli* strain BL21(DE3). Purification of GST fusion proteins was performed as described by Frangioni and Neel (17). Recombinant GCMa-Flag and Flag-CBP proteins were prepared by the baculovirus-insect cell expression system as described previously (10,12). To study the physical interaction between GCMa and HDAC3, GST pull-down experiments were performed by incubating 0.4 μg of GCMa-Flag and 2.5 μg of GST or GST-HDAC3 pre-bound to glutathione beads (Amersham Biosciences) in lysis buffer A at 4°C for 4 h. The beads were then washed five times with lysis buffer A and analyzed by immunoblotting with FLAG mAb. Similar experiments were performed to study the physical interaction between CBP and HDAC3.

To map the interaction domain of GCMa for HDAC3, MBP-HDAC3 or MBP was incubated with GST or a series of GST fusion proteins of full-length or deletion GCMa as indicated in Figure 4B in lysis buffer A at 4°C overnight. To map the interaction domain of CBP for HDAC3, MBP-HDAC3 or MBP was incubated with GST or a series of GST fusion proteins of deletion CBP as indicated in Figure 4B in lysis buffer A at 4°C for 4 h. MBP-GCMa and GST fusion proteins were prepared as described previously (12). Preparation of MBP-HDAC3 was similar to that of MBP-GCMa. After incubation, the beads were washed five times with lysis buffer A and analyzed by immunoblotting with a mouse anti-MBP monoclonal antibody (MBP mAb, Clontech, Palo Alto, CA).

**Chromatin immunoprecipitation (ChIP) assay**

Approximately 3 × 10⁶ BeWo cells were mock-treated or treated with 50 μM forskolin for 24 h or 50 ng/ml of TSA for 12 h before being subjected to a ChIP assay as described previously (12). Associated protein–DNA complex were incubated with HDAC3 (Abcam, Cambridge, UK) or Ac-H3 (recognizing acetylated-K9 and -K14, Upstate, Lake Placid, NY) antibody and then precipitated with protein A-conjugated agarose beads. PCR conditions and primer sequences for a specific region containing the pGBS sequence in the syncytin promoter and for a specific region in the GAPDH promoter have been described previously (12).

**RESULTS**

**TSA increases the transcriptional activity of GCMa**

Recently we have demonstrated that activation of the cAMP/PKA signaling pathway by forskolin leads to CBP-dependent acetylation of GCMa, which stabilizes GCMa and enhances GCMa-mediated transcriptional activation (12). Increased GCMa activity can promote trophoblastic fusion events in cultured placental BeWo cells and may have a similar effect during placental development. Because protein acetylation is a reversible modification, we investigated whether HDACs are involved in the deacetylation of GCMa and thereby the regulation of GCMa activity. To this end, we tested the effect of TSA, a class I and II HDAC inhibitor, on the level of acetylated GCMa in 293T cells transfected with pGal4-GCMa-Flag, pCBP-HA and pPKAcata. As shown in Figure 1A, CBP-mediated acetylation of Gal4-GCMa-Flag was specifically detected because Gal4-Flag was not acetylated in the presence of CBP and Gal4-GCMa-Flag was not acetylated in the absence of CBP. Moreover, the level of acetylated Gal4-GCMa-Flag was further increased by TSA in a dose-dependent manner. We also tested the effect of nicotinamide, a class III HDAC inhibitor, in a similar experiment and did not detect any significant effect on the level of acetylated GCMa (data not shown). These results suggested that deacetylation of GCMa is very likely to be regulated by class I or II HDACs in vivo.

We further investigated the effect of TSA on GCMa transcriptional activity. 293T cells were transfected with different combinations of p(pGBS)E1bLUC, pHA-GCMa, pCBP-HA and pPKAcata, followed by treatment with or without TSA. As shown in Figure 1B, the luciferase activity directed by p(pGBS)E1bLUC was stimulated by GCMa, which could be enhanced by PKA or to a higher degree by a combination of PKA and CBP. Interestingly, the positive effect of PKA alone or PKA and CBP on GCMa transcriptional activity was further stimulated by TSA (Figure 1B). Moreover, the stimulation of GCMa transcriptional activity...
by TSA was dose-dependent in 293T cells expressing HA-GCMa alone or together with PKA and CBP (Figure 1C). These results suggested that inhibition of GCMa deacetylation enhances the transcriptional activity of GCMa.

Identification of HDACs interacting with and deacetylating GCMa and CBP

To identify HDACs involved in GCMa deacetylation, we investigated the protein–protein interaction between GCMa and HDAC1-5 by co-immunoprecipitation. 293T cells were transfected with pHA-GCMa and the indicated pHDAC1-, 2-, 3-, 4- or 5-Myc expression plasmid. After immunoprecipitation using GCMa Ab and immunoblotting using Ac-K mAb, specific interactions were detected between GCMa and HDAC1, 3, 4 and 5 (Figure 2A). Correspondingly, the same result was observed in a similar experiment by immunoprecipitation of HDACs with Myc mAb and immunoblotting with HA mAb (Figure 1C). These results suggested that inhibition of GCMa deacetylation enhances the transcriptional activity of GCMa.

Figure 1. Regulation of GCMa activity by TSA. (A) TSA increases the level of acetylated GCMa. 293T cells were transfected with the indicated combinations of 2 μg of pGal4-Flag, 2 μg of pGal4-GCMa-Flag, 0.1 μg of pPKAcata and 1 μg of pCBP-HA. After 24 h post-transfection, cells were mock-treated or treated with an increasing amount of TSA for another 24 h. The level of acetylated Gal4-GCMa-Flag was analyzed by immunoprecipitation using GCMa Ab and immunoblotting using Ac-K mAb, as described in Materials and Methods. The protein level of Gal4-GCMa-Flag in each transfection group was detected by immunoblotting using FLAG mAb. (B and C) TSA stimulates GCMa-mediated transcriptional activation. 293T cells were transfected with 0.6 μg of p(pGBS)E1bLUC and the indicated combinations of 0 μg of pHA-GCMa, 0.1 μg of pPKAcata and 0.5 μg of pCBP-HA. After 24 h post-transfection, cells were mock-treated or treated with 100 ng/ml of TSA (B) or increasing amounts of TSA (C) for another 24 h. Note that a dose-dependent effect of TSA on GCMa-mediated transcriptional activation was observed. Mean values and SEM obtained from three independent transfection experiments are provided.
Because HDAC1 has been recently reported to recruit p300 HAT (18), we investigated whether HDACs can also interact with CBP. We analyzed the interaction between CBP and HDAC1-5 by co-immunoprecipitation in 293T cells transfected with pCBP-HA and pHDAC1-, 2-, 3-, 4- or 5-Myc. As shown in Figure 3A, specific interactions between CBP and HDAC1 and 3, but not HDAC2, 4 and 5, were detected from using HA mAb for immunoprecipitation and Myc mAb for immunoblotting. These results were also confirmed in a similar experiment using Myc mAb for immunoprecipitation and HA mAb for immunoblotting (Figure 3A). Since the interaction between CBP and HDAC3 is a new finding and may further complicate current understanding of how GCMa activity is regulated, we focused on the roles of HDAC3 in the regulation of CBP-mediated GCMa acetylation for the rest of this study. To examine the interaction patterns between CBP, GCMa and HDAC3 in vivo, we cotransfected 293T cells with pGCMa-Flag, pCBP-HA and pHDAC3-Myc, followed by co-immunoprecipitation using different pairs of FLAG, Myc and HA mAbs as well as normal mouse serum (NS) as a control. As shown in Figure 3B, specific interactions were detected between GCMa and CBP, CBP and HDAC3 and GCMa and HDAC3 using different combinations of FLAG, Myc and HA mAbs, but not NS. We further characterized the aforementioned interaction patterns by sedimentation analysis of immunopurified and eluted CBP–Flag complexes from 293T cells transfected with pHA-GCMa, pCBP-Flag and pHDAC3-Myc. The interaction between HA-GCMa and CBP was instability after the elution of CBP-Flag (data not shown). Nevertheless, the interaction between HDAC3-Myc and CBP-Flag and between HDAC3-Myc and HA-GCMa is relatively stable for further sedimentation analysis. As shown in Figure 3C, the HDAC3–CBP complex and the HDAC3–GCMa complex were detected and located in different fractions (fractions 4 and 5 for the HDAC3–CBP complex and fractions 10, 11 and 12 for the HDAC3–GCMa complex), suggesting that HDAC3 may differentially interact with CBP and GCMa. Although signals for HA-GCMa and HDAC3-Myc were detected in fractions 1 and 16 (Figure 3C), these were likely due to protein aggregation and dissociation of protein complex, respectively.

Identification of the interaction domains of GCMa and CBP for HDAC3

We next performed GST pull-down experiments to verify the direct interaction between GCMa and HDAC3 by incubating recombinant GCMa-Flag protein with recombinant GST or GST-HDAC3 protein. A similar experiment was also performed to verify the direct interaction between CBP and
Figure 3. Characterization of the interaction between HDAC3, GCMa and CBP. (A) CBP interacts with HDAC1 and 3. 293T cells were transfected with the indicated combinations of 1 μg of pCBP-HA, 1.5 μg of pHDAC1-, 3- and 5-Myc, 2 μg of pHDAC2-Myc and 1 μg of pHDAC4-Myc. After 48 h post-transfection, cells were harvested for interaction analysis by co-immunoprecipitation as described in Materials and Methods. Note that results of reciprocal co-immunoprecipitation experiments are presented (upper two panels). The protein levels of CBP-HA and HDAC-Myc in the WCL are presented (lower two panels). (B and C) Analysis of the interaction between CBP, GCMa and HDAC3 by co-immunoprecipitation and sedimentation analysis. (B) 293T cells were transfected with 2 μg of pGCMa-Flag, 2.5 μg of pCBP-HA and 1.5 μg of pHDAC3-Myc. After 48 h post-transfection, cells were harvested for co-immunoprecipitation using the indicated combination of antibodies to detect the level of immunopurified protein and the interaction between the immunopurified protein and the other two co-expressing proteins. NS, normal serum. (C) 293T cells were transfected with 2 μg of pHA-GCMa, 2 μg of pCBP-Flag and 2 μg of pHDAC3-Myc and the CBP–Flag complexes were immunopurified, eluted with FLAG peptide, and subject to 10 to 30% glycerol gradient centrifugation. Each fraction was analyzed by immunoblotting using the HA, FLAG and Myc mAbs for detection of HA-GCMa, CBP-Flag and HDAC3-Myc, respectively.
HDAC3 using recombinant Flag-CBP, GST and GST-HDAC3 proteins. As shown in Figure 4A, GST-HDAC3, but not GST, directly and specifically interacted with GCMa-Flag and Flag-CBP, respectively. We further mapped the interaction domains of GCMa and CBP binding HDAC3. For CBP domain mapping, a series of GST–CBP fusion proteins were incubated with MBP or MBP-HDAC3 for GST pull-down analysis. Specific interactions were detected between HDAC3 and the regions of amino acids 1–451 and 1892–2163 in CBP (Figure 4B, left panel). No interaction was detected when only MBP was incubated with GST–CBP fusion proteins (Figure 4B, left panel). A similar experiment was performed for GCMa domain mapping by incubating a series of GST–GCMa fusion proteins with MBP or MBP-HDAC3. As shown in Figure 4B (right panel), specific interactions were detected between HDAC3
and the regions of amino acids 167–349 and 300–436 in GCMa, suggesting that both regions are involved in interaction with HDAC3.

HDAC3 represses CBP transcriptional coactivator activity and CBP-enhanced GCMa transcriptional activity

Since CBP can be self-acetylated via its own HAT activity and can interact with HDAC3 as described above, we tested whether HDAC3 can deacetylate CBP. 293T cells were transfected with pCBP-HA alone or together with pHDAC3-Myc, followed by acetylation assays using HA mAb for immunoprecipitation and Ac-K mAb for immunoblotting. As shown in Figure 5A, acetylation of CBP was detected, which could be inhibited in the presence of HDAC3. We also tested the effect of HDAC3 on CBP activity in terms of transcriptional coactivation. JEG3 cells were cotransfected with pG5E1bLUC, pGal4-CBP-Flag and increasing amounts of pHDAC3-Flag (0.4 and 0.8 µg) or pHDAC4-Flag (0.025 and 0.05 µg). After 48 h post-transfection, cells were harvested for luciferase assay as described in Materials and Methods. Mean values and the SEM obtained from six independent transfection experiments are presented. (C) HDAC3 and 4 repress the enhancement effect of CBP on GCMa-mediated transcriptional activation. 293T cells were transfected with different combinations of 1.5 µg of p(pGBS)E1bLUC, 0.17 µg of pHA-GCMa, 0.17 µg of pCBP-HA, 0.17 µg of pCBP-HA, 0.17 µg of pHDAC3-Flag and 0.026 µg of pHDAC4-Flag. Mean values and the SEM obtained from six independent transfection experiments are presented. (D) Stabilization of GCMa by CBP was counteracted by HDAC3. 293T cells were transfected with different combinations of 1.5 µg of pHA-GCMa, 0.75 µg of pCBP-Flag and 1.25 µg of pHDAC3-Flag. After 36 h post-transfection, cells were pulsed with [35S]-methionine for 1 h and then chased with cold methionine for the indicated time. Radioactive HA-GCMa proteins were immunoprecipitated by HA mAb, analyzed by SDS–PAGE and fluorography, and quantified by a bioimaging analyzer. Mean values and the SEM from two independent transfection experiments are presented.
impose a stronger inhibitory effect than HDAC4 on CBP-mediated transcriptional coactivation.

We next tested whether HDAC3 and 4 also regulates CBP-enhanced GCMa transcriptional activity. To this end, 293T cells were transfected with different combinations of p(GBS)\textsubscript{E1bLUC}, pHA-GCMa, pcBP-HA and pHDAC3- and 4-Flag. As shown in Figure 5C, the luciferase activity directed by p(GBS)\textsubscript{E1bLUC} was activated by GCMa. Moreover, HDAC3 and 4 did not significantly affect the observed GCMa-mediated transcriptional activation, whereas CBP significantly enhanced it. However, the enhancement effect of CBP on GCMa transcriptional activity was repressed in the presence of HDAC3 or 4 (Figure 5C) when a similar level of HDAC3- or 4-Flag protein was expressed in the transfected cells (data not shown). We were curious about whether HDAC3 counteracts the HAT activity of CBP and imposed the observed repression effect. To this end, a HAT mutant CBP expression plasmid, pCBP\textsuperscript{HAT}\textsubscript{E1C0}-HA, was tested in transient expression experiments. As shown in Figure 5C, consistent with our previous study (12), the enhancement effect of CBP\textsuperscript{HAT} on GCMa transcriptional activity was lower than that of wild-type CBP. Moreover, HDAC3 and 4 did not significantly repress the enhancement effect of CBP\textsuperscript{HAT} (Figure 5C), suggesting that HDAC3 and 4 primarily counteracts the HAT activity of CBP thereby repressing the CBP-enhanced GCMa transcriptional activation. On the other hand, since acetylation of GCMa by CBP increases the stability of GCMa, we examined whether HDAC3 can reverse the stabilizing effect of CBP on GCMa by comparing the half-lives of GCMa in 293T cells transfected with different combinations of pHA-GCMa, pcBP-Flag and pHDAC3-Flag. As shown in Figure 5D, the half-life of GCMa was not affected by HDAC3, but was significantly prolonged in the presence of CBP. However, the stabilizing effect of CBP on GCMa was repressed when GCMa was coexpressed with CBP and HDAC3 (Figure 5D). Therefore, HDAC3 can reverse the stabilizing effect of CBP on GCMa, which is very likely attributed to the above-mentioned repression effect of HDAC3 on the CBP-enhanced GCMa transcriptional activation.

Regulation of GCMa activity by HDAC3 in placental cells

Because sycnytin gene is a direct target gene of GCMa, we now investigated if HDAC3 has any role in the regulation of sycnytin gene expression. To determine the association of HDAC3 with the sycnytin promoter, ChIP assays were performed to analyze the association of HDAC3 with the proximal GCMa-binding site (pGBS) in the sycnytin promoter in mock-, forskolin- and TSA-treated BeWo cells. As shown in Figure 6A, association of HDAC3 with pGBS was detected in mock- or TSA-treated BeWo cells. Interestingly, the association of HDAC3 with pGBS was significantly decreased when BeWo cells were treated with forskolin. As a control, HDAC3 did not associate with a designated promoter region of GADPH (Figure 6A). On the other hand, acetylation of histone 3 (Ac-H3) around the pGBS site was detected and not significantly changed in all three groups of BeWo cells (Figure 6A). Therefore, the association of HDAC3 with the sycnytin promoter is dynamic and is decreased by forskolin, a stimulatory factor for sycnytin gene expression.

We further investigated the role of HDAC3 and 4 in the regulation of sycnytin gene expression by transient expression experiments. To this end, we first tested the effect of TSA on sycnytin promoter activity by transfecting placental JAR cells with pLUC(25468–30953), followed by treatment without or with increasing amounts of TSA for 24 h. After 24 h post-transfection, luciferase activity was measured and the SEM obtained from three independent transfection experiments is presented.

Figure 6. Regulation of sycnytin gene expression by HDAC3 and 4. (A) HDAC3 associates with the pGBS in the sycnytin promoter. BeWo cells were mock-treated or treated with 50 \(\mu\)M forskolin for 24 h or 50 ng/ml of TSA for 12 h and analyzed by ChIP assays for a promoter region covering pGBS in the sycnytin gene or for a promoter region in the GADPH gene. A reaction was performed in the absence of antibody as a control (no Ab). Serial dilutions of input chromatin DNA were analyzed in PCR with primers for the sycnytin promoter. (B) TSA stimulates sycnytin promoter activity and HDAC3 and 4 repress the sycnytin promoter activity activated by GCMa and CBP. JAR cells were transfected with the indicated combinations of 0.1 \(\mu\)g of pLUC(25468–30953), 0.1 \(\mu\)g of pHA-GCMa, 0.1 \(\mu\)g of pCBP-HA and 0.2 \(\mu\)g of pHDAC3- and 4-Flag. After 24 h post-transfection, luciferase activity was measured and the SEM obtained from three independent transfection experiments is presented.
with increasing amounts of TSA. As shown in Figure 6B, the transcriptional activity directed by pLUC(25468–30953) was stimulated by TSA at 15 or 25 ng/ml, suggesting that inhibition of HDAC activity can upregulate syncytin promoter activity. Since it is known that syncytin promoter activity is upregulated by GCMa and CBP (12), we also tested whether HDAC3 and 4 could affect GCMa and CBP-upregulated syncytin promoter activity by transfecting JAR cells with different combinations of pLUC(25468–30953), pCBP-HA, pHa-GCMa and pHDAC3- and 4-Flag. Consistent with our recent findings, GCMa and CBP stimulated the reporter activity directed by pLUC(25468–30953) via the syncytin promoter element (Figure 6B). However, the observed transcriptional activation of syncytin promoter was repressed in the presence of HDAC3 or 4 (Figure 6B). Taken together, these results suggested that HDAC3 and 4 represses the syncytin promoter activity activated by CBP and GCMa.

**DISCUSSION**

Human placental villi are covered with a terminally differentiated multinucleated syncytiotrophoblast layer, which is responsible for most of the transport and endocrine activities of placenta during pregnancy. Syncytin can mediate trophoblastic fusion and is therefore an important effector in the formation of syncytiotrophoblast layer. GCMa regulates syncytin gene expression, which can be further stimulated by the cAMP/PKA signaling pathway with CBP, a transcription coactivator and a HAT, that acetylates GCMa and thereby prolongs the half-life of GCMa. In the present study, we provided evidence that HDAC3 is a key factor that negatively regulates GCMa activity via deacetylation of GCMa. Several lines of evidence support this conclusion. First, the HDAC inhibitor, TSA, elevated the level of acetylated GCMa, suggestive of a functional interaction between HDAC and GCMa. Second, HDAC3 physically interacted with and deacetylated GCMa. Third, the enhancement effect of CBP on GCMa-mediated transcriptional activation was counteracted by HDAC3.

Protein acetylation regulates a variety of protein functions involved in chromatin assembly, protein–protein interaction, stability, DNA-binding activity, transcriptional activity and nuclear localization. Interestingly, a target protein may interact with several HDACs. For example, Stat3 can interact with HDAC1, 2, and 3, and RUNX3 can interact with HDAC1, 2, 4 and 5 (19,20). In addition to HDAC3, we also demonstrated that GCMa co-immunoprecipitates with and is deacetylated by HDAC1, 4 and 5 in the current study. By searching expressed sequence tag (EST) expression profiles in GenBank, we found that HDAC1, 3, 4 and 5 transcripts are expressed in human placenta, suggesting that HDAC1, 3, 4 and 5 are all likely to play a role in the deacetylation of GCMa in placenta. HDAC4 and 5, two class II HDACs, are able to shuttle in and out of the nucleus in response to certain cellular signals as in the case of muscle differentiation in which phosphorylation of HDAC4 and 5 by Ca$$^{2+}$/calmodulin-dependent kinase causes both HDACs to relocalize from the nucleus to the cytoplasm (21). It will be interesting to investigate whether shuttling of HDAC4 and 5 takes place in placenta and regulates GCMa activity.

It is generally believed that the syncytiotrophoblast layer undergoes apoptosis and is replenished by cell–cell fusion of subjacent mononucleated cytotrophoblasts. Therefore, a balance between apoptosis and cell–cell fusion is imperative to maintain the syncytiotrophoblast layer as a steady-state structure in placenta during pregnancy. Apparently GCMa plays a pivotal role in this balancing mechanism because GCMa regulates syncytin gene expression and consequently trophoblastic fusion. Once GCMa activity is increased as induced by cAMP/PKA signaling, it is thought that the dynamics of the syncytiotrophoblast layer will be tilted toward trophoblastic fusion. Should GCMa activity be too high, the steady-state structure of the syncytiotrophoblast layer may be affected in such a way as to cause adverse effects on placental functions. Therefore, a counteracting mechanism to reduce GCMa activity is critical to maintaining normal placental functions. In the present study, we found that HDAC3 associates with pGBS in the syncytin promoter in placental BeWo cells and turns into a dissociated form in the presence of forskolin. In accordance with our previous ChIP studies, that forskolin enhances the association of GCMa and CBP with pGBS, it is a feasible to speculate that CBP displaces HDAC3 from GCMa on pGBS when syncytin gene expression is stimulated by forskolin. The displacement of HDAC3 from GCMa by CBP may involve a direct interaction between CBP and HDAC3 as supported by GST pull-down and sedimentation analyses. On the other hand, when the level of GCMa (most likely in the acetylated form as in the presence of TSA) on pGBS is increased, HDAC3 associates with GCMa (Figure 6A), very likely for the reason of deacetylating GCMa and hence reducing the GCMa activity as ubiquitination of deacetylated GCMa can take place subsequently. Correlatively, we also demonstrated that HDAC3 reduces syncytin promoter activity activated by CBP and GCMa in transient expression experiments (Figure 6B). Together with our previous findings that CBP HAT upregulates GCMa activity, this study supports that HDAC3 plays an important role in counteracting elevated GCMa activity. Therefore, trophoblastic fusion in normal placental morphogenesis may heavily rely on the regulation of GCMa activity by HAT and HDAC.

**ACKNOWLEDGEMENTS**

This work was supported by grants (to H.C.) from the National Science Council (Grant 94-2311-B-001-035) and Academia Sinica, Taiwan. Funding to pay the Open Access publication charges for this article was provided by the National Science Council (Grant 94-2311-B-001-035) of Taiwan.

Conflict of interest statement. None declared.

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