PMA-induced GCMa phosphorylation stimulates its transcriptional activity and degradation

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Abstract
Glial cells missing Drosophila homolog a (GCMa) is a member of the GCM transcription factor family and plays critical roles in trophoblast differentiation and placental functions. It is well established that the cyclic AMP (cAMP)-dependent pathway induces the expression and transcriptional activity of GCMa by regulating post-translational modifications of GCMa, which results in enhancement of trophoblast differentiation. We previously observed that phorbol 12-myristate 13-acetate (PMA) stimulates phosphorylation of GCMa on serines 328, 378 and 383 through the protein kinase C (PKC)- and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)-dependent pathway, which decreases the protein stability of GCMa. Here we report that PMA increases the ubiquitination level of GCMa, dependent on the phosphorylation of GCMa on serines 328, 378 and 383. We found that this phosphorylation also stimulates the transcriptional activity of GCMa. Our data indicate that the PMA-induced PKC- and MEK/ERK-dependent pathway enhances the degradation as well as the transcriptional activity of GCMa. We also examined the impact of this signaling pathway on trophoblasts and the results suggest that the PKC- and MEK/ERK-dependent pathway is involved in the regulation of trophoblast differentiation.

In mammals, two GCM (glial cells missing) proteins, GCMa and GCMb (also known as GCM1 and GCM2) have been identified (24, 25). The first-identified drosophila GCM was identified as a gene responsible for a phenotype called glial cells missing, which is the gene name GCM derived (19, 23). GCMa and GCMb are predominantly expressed in developing placenta and the parathyroid gland, respectively (5, 16, 29). GCMa instructs trophoblast stem cells to differentiate into syncytiotrophoblast cells (1, 2). Abnormal development of placenta causes embryonic death in GCMa knockout mice (33). GCMa is also related to the control of placental functions by regulating gene expressions of aromatase, syncytin, and placental growth factor (8, 37, 40). Thus GCMa is essential for normal placental development and functions, and abnormalities in GCMa expression may be associated with pregnancy complications in humans, as decreased levels of GCMa expression have been reported in pre-eclampsia cases (4, 9).

Recent studies have shown that the expression level and transcriptional activity of GCMa are regulated by its post-translational modifications. The acetylation and deacetylation by CRE-binding protein-binding protein (CBP) and histone deacetylase 3 (HDAC3) regulate the protein stability of GCMa (7, 13), and the sumoylation destabilizes the interaction between GCMa and the specific DNA sequence called GCM motif (6, 12). The phosphorylation of GCMa on serine 322 results in its ubiquitination and degradation (11). The phosphorylation on serines 269
and 275 enhances the interaction between GCMA and dual-specificity phosphatase 23 that dephosphorylates phosphorylated serine 322 (28). Thus post-translational modifications regulate functions of GCMA in diverse steps.

We have shown that phorbol 12-myristate 13-acetate (PMA) stimulates the phosphorylation of GCMA on serines 328, 378 and 383 through a protein kinase C (PKC)- and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)-dependent pathway, and we described the possibility that this phosphorylation enhances GCMA degradation (39). It has been indicated that phosphorylation on a transcription factor influences its transcriptional activity. For example, nuclear factor (NF)-κB is phosphorylated on the p65 subunit, which stimulates its transcriptional activity (41). In contrast, STAT6 (signal transducers and activation of transcription 6) is inactivated by its phosphorylation (34). However, the questions of how phosphorylation of GCMA affects its degradation process and whether it affects the transcriptional activity of GCMA are as yet unanswered.

In the present study, we examined the effects of the phosphorylation of GCMA on serines 328, 378 and 383 by the PMA-induced PKC- and MEK/ERK-dependent pathway on the ubiquitination and transcriptional activity of GCMA. We also investigated whether this signaling pathway is related to human chorionic gonadotropin (hCG) production in syncytiotrophoblasts, to evaluate the influence of the PKC- and MEK/ERK-dependent pathway on trophoblast differentiation. Our data demonstrated that the PMA-induced PKC- and MEK/ERK-dependent pathway enhances the transcriptional activity of GCMA. Furthermore, this pathway also increases GCMA degradation by upregulating its ubiquitination. These effects appear to be involved in the regulation of trophoblast differentiation.

MATERIALS AND METHODS

Reagents. PMA, dimethyl sulfoxide (DMSO), forskolin (FSK) and U0126 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ro-31-8220 and epidermal growth factor (EGF) were purchased from Biomiol International (Plymouth Meeting, PA) and HIGETA SHOYU Co. (Tokyo), respectively. Antibodies against DYKDDDDK (FLAG-tag), ubiquitin, p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (pERK1/2) were purchased from Cell Signaling Technology (Beverly, MA). Characterization of anti-GCMA antibody was described previously (39).

Cell culture. JEG-3 and HEK293T cells were obtained from the American Type Culture Collection (Manassas, MD). JEG-3 cell line is a human choriocarcinoma that expresses endogenous GCMA. HEK293T cell line is derived from human embryonic kidney containing the SV40 large T-antigen. JEG-3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mM sodium pyruvate, 1% MEM Non-Essential Amino Acids (all from Wako Pure Chemical Industries) and 10% fetal calf serum (FCS) at 37°C in 5% CO₂. HEK293T cells were cultured in DMEM containing 10% FCS at 37°C in 5% CO₂.

Plasmid construction. FLAG-tagged expression plasmids for wild-type or mutant of GCMA were described previously (39). GCMA-luciferase reporter plasmid was constructed by subcloning the five tandem repeat sequence of GCMA motif (GACCCTCA TT) into pGL4.26 (Promega, Fitchburg, WI) (37).

Immunoprecipitation. HEK293T cells were plated into 24-well plates at a density of 1.0 × 10⁵ cells/well the day before transfection. The cells were transfected with 0.5 μg of GCMA expression plasmid using 1 μL of FuGENE HD as indicated above. After 24 h, the medium was changed into FCS-free...
medium and the cells were treated with 0.1% DMSO or 10 μM of PMA for 30 min. As described previously (39), cell lysates were separated by SDS-PAGE using 8% acrylamide gels including 15 μM Phos-tag acrylamide (Nard Chemicals, Hiroshima, Japan) and 30 μM MnCl₂. After electrophoresis, the gels were washed in transfer buffer containing 1 mM EDTA for 10 min and then transferred onto PVDF membranes as described (26). The membranes were treated, and the bands were visualized as described above. The phosphorylated GCMa was detected as a shifted band on the immunoblot.

**GCMa-dependent luciferase assay.** HEK293T cells were plated into 24-well plates at a density of 2.0 × 10⁵ cells/well the day before transfection. The cells in 500 μL of normal medium were transfected with GCMa expression plasmid (0.25 μg), GCM-luciferase reporter plasmid (0.25 μg) and reference plasmid (pGL4.73) (0.001 μg) using 1 μL of FugeneHD (Promega). At 17 h after transfection, the medium was changed into FCS-free medium and the cells were treated with 10 μM PMA with or without 200 nM Ro-31-8220 or 20 μM U0126 for 6 h. The cells were then harvested and the luciferase activity was determined with a Dual-Luciferase Reporter Assay system kit (Promega).

**hCG measurement by enzyme-linked immunosorbent assay.** JEG-3 cells were plated into 24-well plates at a density of 1.5 × 10⁵ cells/well the day before transfection. The medium was changed to FCS-free DMEM containing 1 mM sodium pyruvate and 1% MEM non-essential amino acids. The cells were treated with 0.1% DMSO or 10 μM PMA, plus 200 nM Ro-31-8220 or 20 μM of U0126 for 24 h, and then the medium was collected. The hCG content in the medium was measured using Chorionic Gonadotropin, Human, ELISA kit (Immunospec Corp., Chatsworth, CA) in accordance with the manufacturer’s instruction.

**RESULTS AND DISCUSSION**

**PMA increases the ubiquitination level of GCMa through its phosphorylation on serines 328, 378 and 383**

Our previous study indicated that PMA induces GCMa phosphorylation on serines 328, 378 and 383, and the phosphorylation stimulates its degradation; the GCMa mutated on all the three serines is less susceptible to its degradation compared to wild-type of GCMa (39). Since it has been shown that GCMa is degraded by a ubiquitin-proteasome system (10, 38), we investigated here whether the phosphorylation of GCMa on these serines influences its ubiquitination level. To examine the ubiquitination of GCMa, FLAG-tagged GCMa wild-type or mutant S328,378,383A was expressed in HEK293T cells (Fig. 1A). The cell lysates were immunoprecipitated using anti-GCMa antibody to concentrate GCMa or its mutant protein (Fig. 1B, lower panel). Resulting immunoprecipitants were analysed for its ubiquitination level by immunoblot using anti-Ubiquitin antibody. As shown in Figure 1B, upper panel, ubiquitination level of GCMa was upregulated by PMA treatment, but that of the mutated GCMa on serines 328, 378 and 383 was not. These data suggest that PMA-induced phosphorylation of GCMa on serines 328, 378 and 383 promotes GCMa degradation by upregulating its ubiquitination level.

**The transcriptional activity of GCMa increases through the PKC- and MEK/ERK-dependent pathway in the PMA-treated cells**

It has been shown that post-translational modification of transcription factor is related in its transcriptional activity (14, 21, 34, 41). Here we investigated whether the PMA-induced phosphorylation of GCMa has an impact on its transcriptional activity. As shown in Figure 2A, we first tested whether phosphorylation of GCMa was induced by PMA treatment in a PKC- and MEK/ERK-dependent manner in HEK293T cells, as well as in JEG-3 cells as described previously (39). We then studied how PMA influences GCMa transcriptional activity, using a luciferase reporter assay. To this end, HEK293T cells were transiently transfected with the GCMa expression plasmid and GCM-luciferase reporter plasmid, and then treated with PMA with or without PKC inhibitor, Ro-31-8220 or MEK inhibitor, U0126. The results indicated that GCMa-dependent luciferase activity was enhanced by PMA treatment, which was inhibited by PKC or MEK inhibitor (Fig. 2B). These data suggest the possibility that PMA-induced phosphorylation of GCMa activates its transcriptional activity through the PKC- and MEK/ERK-dependent pathway.

**Phosphorylation of GCMa on serines 328, 378 and 383 contributes to the PMA-induced transcriptional activity of GCMa**

Since we observed that serines 328, 378 and 383 are phosphorylated by PMA treatment, we next examined the relation between phosphorylation of these serines and transcriptional activity in GCMa.
In this experiment, HEK293T cells were co-transfected with GCMLuciferase reporter plasmid and an expression plasmid of several GCMa mutants whose serine(s) is converted into alanine(s) and treated with or without PMA, and then luciferase assay was performed. The results show that the increase in the number of the mutated sites on GCMa attenuated its luciferase activity activated by PMA treatment (Fig. 3). These data suggest that phosphorylation of GCMa on the three serines contributes to the activation of its transcriptional activity by PMA treatment. However, the transcriptional activity of GCMa mutant on all three serines was still activated by PMA treatment, and therefore we predict that there are other mechanisms underlying the way(s) that PMA enhances transcriptional activity of GCMa independent of GCMa phosphorylation.

Since serine 328 is located on the nuclear localization signal sequence and serines 378 and 383 are on the transactivation domain, we thought about the possibility that the intracellular localization or transcriptional function of GCMa may be affected by phosphorylation. However, the contributions of these serines to the transcriptional activity of GCMa seem to be equal. As shown in Figure 3, the phosphorylation sites have additive effects on the transcriptional activity of GCMa, though mutation in two of the three serines appears to be enough to decrease it. The details are unclear as to how the PMA-initiated phosphorylation works to enhance the transcriptional activity of GCMa, but these serines probably do not enhance its transcriptional activity independently, as is the case for the phosphorylation of NF-κB p65 subunit on serine 276 or STAT6 on serine 707.

![Fig. 1](image)

**Fig. 1** PMA increases the ubiquitination level of GCMa, which is attenuated by mutation of GCMa on serines 328, 378 and 383 into alanines. HEK293T cells were transfected with expression plasmid of Barnase (Control), wild-type (GCMa WT) or triple mutant on serines 328, 378 and 383 (S328,378,383A) of GCMa and then treated with DMSO (0.1%) or PMA (10 μM). The cell lysates were immunoprecipitated using anti-GCMa antibody. The resulting proteins were analyzed by immunoblot using anti-tag or anti-Ubiquitin antibody. **(A)** FLAG-tagged proteins were detected in the whole cell lysates (Input, IB:FLAG). The arrowheads point to FLAG-tagged Barnase (open) and wild-type and mutant of GCMa (filled). **(B)** Those FLAG-tagged GCMa but Barnase are detected in the immunoprecipitants (IP:GCMa, IB:FLAG), in which the GCMa ubiquitination level was increased by PMA treatment, and the mutation on those serines on GCMa moderates the PMA-induced ubiquitination (IP:GCMa, IB:Ubiquitin).
Stimulation of GCMa transcriptional activity

(34, 41). Our data showed that phosphorylation of GCMa on serines 328, 378 and 383 stimulates not only the transcriptional activity but also the degradation of GCMa. We think a possibility that three phosphorylations are likely to cause conformational changes of GCMa as demonstrated in other proteins (15, 31). Phosphorylated GCMa may be active for the transcriptional activity, but at the same time likely to be targeted by ubiquitin-proteasome system. Coincidence of transcriptional activation and degradation enables rapid regulation of GCMa transcriptional activity, which should be of advantage to control the GCMa function strictly. Similar results have been observed in the phosphorylation of AML1 transcription factor (also known as RUNX1). The expression level of AML1 is regulated by acetylation controlled by p300/CBP and HDAC, as well as GCMa (3, 22). Although the function of phosphorylation is dependent on the site(s), the phosphorylation of AML1 on serines 249 and 266 changes the interaction between AML1 and the corepressor mSin3A, which causes activation of transcriptional activity and degradation of AML1 (17, 20). We suspect the phosphorylation of GCMa may be related to an interaction between GCMa and the other protein(s), leading to enhancement of its degradation and transcriptional activity.

The PMA-induced PKC- and MEK/ERK-dependent pathway may enhance trophoblast differentiation into syncytiotrophoblasts

It was suggested that GCMa is a master regulator of the trophoblast differentiation into syncytiotrophoblast (18, 36). In the trophoblast cells, the activation or the increase of GCMa enhances syncytiotropho-

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**Fig. 2** PMA stimulates the phosphorylation and transcriptional activity of GCMa. (A) Phosphorylation of GCMa was stimulated by PMA treatment through PKC- and MEK/ERK-dependent pathway in HEK293T cells. HEK293T cells were transfected with expression plasmid of GCMa (GCMa WT) and then treated with PMA (10 μM) with or without PKC inhibitor, Ro-31-8220 (Ro-31) (200 nM) or MEK inhibitor, U0126 (20 μM). The cell lysates were separated using Phos-tag and normal acrylamide gels, and analyzed by immunoblot using anti-GCMa antibody and anti-phosphoERK (pERK) or anti-ERK (ERK) antibody, respectively. (B) Transcriptional activity of GCMa was stimulated by PMA treatment through the PKC- and MEK/ERK-dependent pathway. HEK293T cells were transfected with GCM-luciferase reporter plasmid and wild-type or indicated point mutant expression plasmid of GCMa and then treated with PMA (10 μM) with or without Ro-31-8220 (Ro-31) (200 nM) or U0126 (20 μM). The cell lysates were used to measure the luciferase activity.

**Fig. 3** Phosphorylation of GCMa on serines 328, 378 and 383 is involved in the enhancement of transcriptional activity of GCMa by PMA treatment. HEK293T cells were transfected with GCM-luciferase reporter plasmid and wild-type or indicated point mutant expression plasmid of GCMa and then treated with DMSO (10%) (open bars) or PMA (10 μM) (filled bars). The cell lysates were used to measure the luciferase activity.
blast formation, accompanied by cell fusion and production of hCG. Our present data indicate that the transcriptional activity and degradation of GCMa are activated through the PMA-induced PKC- and MEK/ERK-dependent pathway. We then investigated how the PKC- and MEK/ERK-dependent pathway affects the trophoblast differentiation. To determine the influence of this pathway on trophoblast differentiation, we used JEG-3 cells which is a well-established human choriocarcinoma cell line that expresses endogenous GCMa and secretes hCG in response to various stimulants (30, 32). We treated JEG-3 cells with PMA with or without Ro-31-8220 or U0126 inhibitor and determined the amount of hCG production. The results showed that PMA increased hCG production by nearly twofold, and the production was repressed by PKC or MEK inhibitor (Fig. 4A). Suzuki et al. reported that the PKC- and MEK/ERK-dependent pathway stimulates the differentiation of human choriocarcinoma BeWo cells, which is consistent with our results (35). We also found that FSK, an activator of the cAMP-dependent pathway, induced the hCG production by nearly 12-fold (Fig. 4B). These data suggest that in JEG-3 cells the PMA-induced PKC- and MEK/ERK-dependent pathway stimulates hCG production but much less so than the FSK-induced cAMP-dependent pathway. The cAMP-dependent pathway has been shown to increase the expression level and transcriptional activity of GCMa in trophoblast cells (6, 7, 27), leading to trophoblast differentiation. In contrast, the PKC- and MEK/ERK-dependent pathway enhances GCMa degradation as well as transcriptional activity. Therefore, its stimulation of hCG production seems to be small. In addition, our result showed that EGF stimulated the hCG production through the MEK/ERK-dependent pathway at the same level as PMA (Fig. 4A), which is consistent with the finding that EGF’s effect on GCMa is similar to that of PMA as we described previously (39). For these reasons, we think that the MEK/ERK-dependent pathway may both set a limit or keep a certain level of GCMa activity and regulate the level of trophoblast differentiation, and since syncytiotrophoblasts are surrounded by maternal blood, this mechanism may be induced by EGF under physiological conditions.

Here we have indicated the possibility that the PKC- and MEK/ERK-dependent pathway stimulates trophoblast differentiation by regulating GCMa activity. Although the detailed mechanisms underlying the regulation of GCMa activity by the PKC- and MEK/ERK-dependent pathway are still unclear, this pathway likely has important roles in the regulation of trophoblast differentiation and functions. Dysfunction of GCMa is implicated in the pathogenesis of pregnancy complications such as pre-eclampsia. Gaining a better understanding of the regulatory mechanisms of GCMa activity will contribute to the knowledge of how signaling pathways are related to the trophoblast differentiation and pregnancy complications.
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