Possible role of the exchange protein directly activated by cyclic AMP (Epac) in the cyclic AMP-dependent functional differentiation and syncytialization of human placental BeWo cells

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BACKGROUND: The mononuclear villous cytotrophoblast (CTB) differentiates and fuses to the multinucleated syncytiotrophoblast (STB), which produces hCG and progesterone. cAMP-mediated intracellular pathways are involved in the process of endocrine differentiation and fusion (syncytialization). The exchange protein directly activated by cAMP (Epac) is a mediator of cAMP signaling. We examined the differential roles of Epac and protein kinase A (PKA) signaling in the cell fusion and differentiation of trophoblast-derived BeWo cells.

METHODS: Epac1 and Epac2 were localized in human placental tissue (n = 9) by immunohistochemistry. The PKA-selective cAMP analog (N6-phenyl-cAMP, Phe) or Epac-selective cAMP analog (CPT) was tested for effects on hCG and progesterone production, and syncytialization in BeWo cells. The effect of knockdown of Epac or its downstream target molecule (Rap1) on syncytialization was evaluated.

RESULTS: Epac1 and Epac2 proteins were expressed in villous CTB, STB, stroma, blood vessels and extravillous CTB of the placenta. Phe increased the expression of hCGa/b mRNA and secretion of hCG protein in BeWo cells (P < 0.01 versus control). CPT-stimulated production of hCG (P < 0.05), albeit to a lesser extent than Phe. Progesterone production was also enhanced by Phe or CPT (P < 0.01 and P < 0.05, respectively). CPT or a stable cAMP analog (dibutyryl-cAMP: Db) increased the number of syncytialized BeWo cells (P < 0.01), whereas Phe did not stimulate fusion. CPT- or Db-induced syncytialization was observed, even in the presence of a PKA inhibitor. Knockdown of Epac1 or Rap1 repressed the Db-, CPT- or forskolin-induced cell fusion.

CONCLUSIONS: The Epac signaling pathway may be associated with the cAMP-mediated functional differentiation and syncytialization of human trophoblasts.

Key words: Epac / syncytialization / hCG / progesterone / BeWo

Introduction

The placenta is a transiently formed multifunctional organ that is mainly composed of the fetal chorion and endometrial decidua. The major role of the placenta is to establish a crosstalk between the maternal and fetal circulations, which permits exchanges of gas and nutrition that are necessary for fetal development. In addition, the placenta functions as an endocrine tissue that produces various steroids, peptide hormones, growth factors and cytokines, which are crucial for the establishment and maintenance of pregnancy. The multinucleated syncytiotrophoblast (STB) layer, which covers floating villi in the placenta, is the interface cell layer that separates fetal and maternal blood (Benirschke and Kaufmann, 2000). The formation of the multinucleated STB layer via cell fusion of mononuclear cytotrophoblast (CTB) is referred to as syncytialization, which is a well-characterized morphological sign of their terminal differentiation. As STB lacks the ability of DNA replication, the layer is maintained by continuous CTB→CTB or CTB→STB cell fusion and apoptosis (Huppertz and Kingdom, 2004). The insufficient development of trophoblast syncytialization may cause pathological conditions, such as pre-eclampsia...
The Epac-selective cAMP analog [8-(4-chlorophenylthio)-2′,6′-diamino-3′-isopropylcAMP (CPT) and the PKA-selective cAMP analog (N6-phenyl-cAMP, Phe) were purchased from the Biolog Life Science Institute (Bremen, Germany). Dibutyryl-cAMP (Db) and FSK were from Sigma-Aldrich (St. Louis, MO, USA). All cAMP analogs were dissolved in water (50 mM stock solution), H89, which is a PKA inhibitor, was purchased from the D. Western Therapeutics Institute, Inc. (Nagoya, Japan) and dissolved in dimethylsulfoxide (10 mM stock solution, Wako Pure Chemical Industries Ltd., Osaka, Japan).

Tissue samples and cell culture

Normal placental tissues of first trimester (6 and 7 weeks of gestation; n = 2 each) and third trimester (37 and 38 weeks of gestation; n = 2 each) were obtained with informed consent from women undergoing surgery, such as Cesarean section. The use of these tissues in the experiments was approved by the clinical research ethics committee of the Tokyo University of Pharmacy and Life Sciences. The human choriocarcinoma cell line BeWo was purchased from the American Type Culture Collection (Manassas, VA, USA) and grown at 37°C in Ham’s F-12/Dulbecco’s modified Eagle’s medium (1:1) medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml neomycin and 0.25 µg/ml amphotericin B.

RNA isolation and RT–PCR analysis

Total RNA was extracted using Isogen (Nippon Gene, Tokyo Japan), according to the manufacturer’s instructions. The total RNA (100 ng) was subjected to real-time RT–PCR using the iScript One-Step RT–PCR Kit with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA). The reactions were carried out on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). The sense (S) and antisense (AS) primers used for real-time RT–PCR were as follows: 5′-TCCACTCTCCTAAGGTCTCAA-3′ (S), 5′-CCCTTATTCTCTGTACCTGGTT-3′ (AS) for hCG; 5′-GCTACTGGCCACCATGGACC-3′ (S), 5′-ATGGAGCTCAAGCGCACATC-3′ (AS) for hCGβ, 5′-CACCTTCCACTGTCAGAA-3′ (S), 5′-ATAAACCCGACTCAGGTC-3′ (AS) for P450scc (P450 side-chain cleavage enzyme) and 5′-AGCCACATCGTCAAGACA-3′ (S), 5′-GGCAATACGACCAATCC-3′ (AS) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change in expression of each gene was calculated using the ΔΔCT method using GAPDH as an internal control.

Immunoblotting

The harvested culture medium from BeWo cells was immediately stored at −30°C until hCGB immunoblotting could be performed. Cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology, Inc., Beverly, MA, USA), according to the manufacturer’s instructions. Equal amounts of medium or lysate protein were subjected to sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were incubated with antibodies against hCGB (clone INN-hCG-22, I:500; AbD Serotec, Oxford, UK), Epac1 (catalog No. ab21235, I:500; Abcam, Cambridge, MA, USA), Epac2 (clone 5B1, I:1000; Cell Signaling Technology) or Rap1 (catalog No. 07-916, I:1000; Upstate biotechnology, Lake Placid, NY, USA) at room temperature for 2 h. Immunoreactive bands were detected using enhanced chemiluminescence (PerkinElmer Life Science, Inc., Boston, MA, USA) after incubation with horseradish peroxidase-labeled goat anti-mouse or rabbit immunoglobulin (Ig)G antibodies (0.5 µg/ml, Vector Laboratories, Burlingame, CA, USA). The membrane was treated with a stripping solution (25 mM glycine–HCl, pH 2.0, containing 1% (w/v) SDS) and re-probed with antibody against GAPDH (clone GAPDH-71.1, I:5000; Sigma-Aldrich). The relative band intensity of hCG was assessed by densitometric analysis of digitalized electrophoresis gels.
autographic images using the Scion Image software (Scion Corp., Fredrick, MD, USA) and normalized to the amount of total cell protein.

**Progesterone assay**

The concentration of progesterone in the culture medium was measured by radioimmunoassay (RIA), as described previously (Tamura et al., 1991) and normalized to the amount of total cell protein.

**Treatment with small interfering RNA**

BeWo cells grown in 24-well culture plates to ~60% confluency were transfected with a non-targeting control small interfering RNAs (siRNAs) (20 pmol/well; catalog No. 1027280, Allstars negative control, Qiagen, Mississauga, ON, Canada), Epac1 (catalog No. sc-41700), Epac2 (sc-41702) or Rap1 (sc-36384) siRNAs (20 pmol/well; Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the Lipofectamine RNAmax transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. After treatment for 24 h with the siRNA, the medium containing siRNA and transfection reagents was removed and cells were cultured for 24 h in fresh culture medium.

**Immunohistochemistry**

The placental tissues of the first trimester and third trimester were fixed in 4% paraformaldehyde before being processed and embedded in paraffin following standard procedures (Tamura et al., 2003). Sections of paraffin-embedded second-trimester placenta (22 weeks of gestation; n = 1) were purchased from Super Bio Chips Laboratories (#SF88122A, Seoul, South Korea). Paraffin sections were rehydrated, boiled for 20 min with 10 mM citrate buffer (pH 6.0), and then blocked with 10% normal goat serum in phosphate-buffered saline for 2 h at room temperature. Sections were incubated with polyclonal anti-Epac1 (catalog No. ab21235, 1:100, Abcam) or anti-Epac2 (clone H-220, 4% paraformaldehyde before being processed and embedded in paraffin) antibodies overnight at 4 °C and were incubated subsequently with amino acid polymer conjugated with anti-rabbit IgG Fab′ and horseradish peroxidase (Histofine Simple Stain MAX-PO MULTI, Nichirei, Tokyo, Japan) and developed with Histofine Simple Stain DAB solution (Nichirei). Negative controls were incubated with rabbit IgG instead of primary antibody. Sections were counterstained with methyl green.

**Cell-fusion assay**

A cell-fusion assay of BeWo cells was performed as described previously (Yoshie et al., 2008). Briefly, BeWo cells grown on poly-L-lysine-coated glass cover slips were fixed and stained with anti-desmosomal protein antibody (clone ZK-31, 1:200, Sigma-Aldrich) together with an AlexaFluor 594 goat anti-mouse antibody (Molecular Probes, Carlsbad, CA, USA) to distinguish cell borders. The nuclei were counterstained with 4′,6-diamino-2-phenylindole 2HCl (DAPI). The number of multinuclear cells in the five microscopic areas that were selected randomly was counted. Data were expressed as the ratio of each control and the effects of selective cAMP analogs, and knocking down of Epac1, Epac2 or Rap1 on syncytialization were evaluated in four independent experiments.

**Statistical analysis**

Data were expressed as mean ± SEM. Significance was assessed using Turkey–Kramer multiple-comparisons testing. A P-value <0.05 was considered statistically significant.

**Results**

**Localization of Epac1 and Epac2 in human placenta**

Expression of Epac1 and Epac2 in placental tissue at different stages was examined by immunohistochemistry. In the first- and second-trimester placenta, Epac1 was expressed in villous CTB, STB, stroma and extravillous trophoblast (EVT) (Fig. 1A and C). In addition to the positive staining of Epac1 in various trophoblasts, blood vessels and stroma in chorionic villi were also stained for this protein (Fig. 1C). In the third trimester of gestation, a low level of staining for Epac1 was detected in STB, blood vessels and EVT (Fig. 1E). Intense staining of Epac2 was detected in villous CTB, stroma and EVT in first- and second-trimester placentas compared with its expression in STB (Fig. 1B and D). Furthermore, Epac2 was expressed in blood vessels (mainly in endothelium) (Fig. 1D). In the third-trimester placenta, Epac2 was observed in STB, blood vessels, and EVT (Fig. 1F). Non-specific immunostaining was not detected in negative control (Fig. 1G).

**Effects of Epac-selective cAMP analog on the production of hCG and progesterone in BeWo cells**

Differentiated STB secretes hCG and progesterone. It is well known that the enhancement of intracellular cAMP levels stimulates the synthesis and secretion of hCG and progesterone (Feinman et al., 1986). We examined the effect of the Epac-selective cAMP analog (CPT) and of the PKA-selective cAMP analog (Phe) on hCG and progesterone production in BeWo cells, a well-characterized trophoblast-derived human choriocarcinoma cell line, which retains the ability to undergo differentiation and syncytialization. Treatment with Phe caused enhancement of hCGα and hCGβ mRNA expression compared with control (P < 0.01), as assessed using quantitative RT–PCR analysis (Fig. 2A and B). CPT increased hCGα and hCGβ mRNA expression moderately (P < 0.05, Fig. 2A and B). However, when compared with control with Phe alone, cotreatment with CPT and Phe did not affect the expression of hCGα and the expression of hCGβ was attenuated (Fig. 2A and B). Changes in hCG protein levels were basically similar to the results obtained for the hCGα and hCGβ mRNA levels, when hCG levels in cultured medium were analyzed using immunoblotting (Fig. 2C). We next examined the effect of CPT and Phe on the production of progesterone in BeWo cells. The culture media used in Fig. 2C were subjected to RIA to determine progesterone levels (Fig. 3A). Compared with control, the content of progesterone was increased by stimulation with CPT (P < 0.05) or Phe (P < 0.01). Simultaneous treatment with Phe and CPT enhanced the production of progesterone further when compared with the Phe treatment alone (P < 0.05). The mRNA level for P450scc, which is a key enzyme for progesterone synthesis that converts cholesterol to pregnenolone, was up-regulated after treatment with Phe (P < 0.01, Fig. 3B). Treatment of CPT caused a 2.1-fold, but not significant, increase in the P450scc mRNA level. However, the level of P450scc mRNA after cotreatment with CPT and Phe was similar to that observed for Phe treatment alone at the same time point (Fig. 3B). We confirmed that the CPT treatment used in this study did not
Figure 1  Expression of the Epac1 and Epac2 in human placenta. Placental tissues at different stages were immunostained with an anti-Epac1 (A, C and E; brown) or Epac2 (B, D and F; brown) antibody. (A, B and G) First trimester; (C and D) Second trimester; (E and F) Third trimester. Nuclei were counterstained with methyl green. (G) Negative control incubated with rabbit immunoglobulin G instead of primary antibody. The squared area in each picture is the magnification of EVT. CTB, cytotrophoblast; STB, syncytiotrophoblast; EVT, extravillous trophoblast; VS, villous stroma; BV, blood vessel. Scale bars = 50 μm.
affect phosphorylation of the cAMP response-element-binding protein, which is a classical downstream target of PKA signaling (data not shown).

Effects of Epac-selective cAMP analog on the syncytialization of BeWo cells

Mononuclear villous CTB continuously fuses and differentiates to form the multinuclear STB layer. Enhancement of intracellular cAMP levels induces their syncytialization in primary isolated trophoblasts (Keryer et al., 1998) and BeWo cells (Ogura et al., 2000). To examine whether Epac-mediated cAMP signaling is involved in syncytialization, BeWo cells were treated with CPT, Phe or a stable cAMP analog (Db) for 48 h. In agreement with the previous report mentioned above, Db increased the ratio of syncytialization of BeWo cells (Fig. 4A and B). In particular, CPT promoted cell fusion significantly ($P < 0.01$), whereas Phe had little effect on syncytialization (Fig. 4A and B). To evaluate the possible relevance of Epac on syncytialization, we examined the effect of CPT- or Db-induced cell fusion in the presence or absence of the PKA inhibitor H89. BeWo cells were pretreated with H89 and were then incubated with CPT or Db for 48 h (Fig. 4C). Vehicle-treated cells served as a control group. CPT or Db treatment elevated syncytialization significantly versus control, even in the presence of the PKA inhibitor, while the ratio of syncytialized cells with Phe was comparable to that observed in the vehicle-treated group (Fig. 4C).

Effects of Epac and Rap1 knockdown on the cAMP-induced syncytialization of BeWo cells

To explore the role of Epac-mediated cAMP signaling on the syncytialization of BeWo cells, we examined the effects of siRNA-mediated knockdown of Epac1, Epac2 or their putative downstream factor, Rap1, on Db-, CPT- or an activator of adenylate cyclase, FSK-induced syncytialization (Fig. 5). Immunoblot analyzes showed that transfection of Epac1, Epac2 or Rap1 specific siRNA repressed expression of each protein by ~60, 80 and 90%, respectively (Fig. 5A). Control siRNA-transfected cells were syncytialized after treatment with Db, CPT or
FSK. However, knocking down of Epac1 or Rap1 resulted in a significant reduction of Db-, CPT- or FSK-induced syncytialization (Fig. 5B and C). In contrast, knockdown of Epac2 did not affect Db-, CPT- or FSK-stimulated syncytialization (Fig. 5B and C).

**Discussion**

The present study demonstrated for the first time that Epac1 and Epac2 were expressed in placental trophoblasts in chorionic villi and that Epac had possible roles in cAMP-mediated functional differentiation and syncytialization. The expression of the Epac1 mRNA in human placenta was confirmed previously using northern blotting (Kawasaki et al., 1998); however, the localization of Epac1 and Epac2 in this tissue has not been reported to date. In first- and second-trimester placenta, both Epac1 and Epac2 were expressed in villous STB, CTB, stroma, blood vessels and EVT. In term placenta, Epac1 and Epac2 were mainly distributed in the STB layer, EVT and blood vessels. Interestingly, Epac1 and Epac2 were localized at the plasma membrane and in the cytoplasm of BeWo cells, respectively (data not shown). Epac1 is translocated rapidly and reversibly to the plasma membrane and activates Rap in response to a cAMP stimulus (Ponsioen et al., 2009). We did not gather definitive information on the significance of intracellular localizations of Epac1 and Epac2 in this study; therefore, further experiments are required to clarify this issue.

The PKA-mediated cAMP signaling pathway is required for hCG synthesis (Chou, 1980; Nulsen et al., 1998; Lambot et al., 2005). Accordingly, the significant increase of hCG production was accompanied by the up-regulation of both hCGα and hCGβ mRNAs after treatment with a PKA-selective cAMP analog (Phe). These results suggest that PKA was the dominant mediator of cAMP-induced hCG synthesis. Our observation of moderate stimulation of the Epac-mediated hCG production suggests that Epac-mediated signaling may also be associated with hCG production. The effect of CPT on hCG production was also observed in another choriocarcinoma cell line, JEG-3 (data not shown). However, CPT did not up-regulate hCG synthesis additively in the presence of Phe; rather, it decreased the levels of the hCGβ mRNA. The Epac pathway might hamper the Phe-mediated hCG production, as the PKA-mediated pathway has been demonstrated to cross-talk with the Epac Pathway (Harper et al., 2008). In addition, Phe increased the level of P450scc mRNA and the production of progesterone significantly. The expression of P450scc is up-regulated by cAMP analogs in primary isolated trophoblast and choriocarcinoma cell lines, which include BeWo cells and JEG-3 cells (Ringler et al., 1989; Martínez et al., 1997). Our results support the notion that PKA is the main mediator of the cAMP-induced P450scc expression. The stimulatory effect of CPT on progesterone secretion was also observed in JEG-3 cells (data not shown). In support of the present study, Chin and Abayasekara (2004) demonstrated that an Epac-selective cAMP analog increases progesterone secretion in human luteinized granulosa cells in a dose-dependent manner. Thus, Epac may be involved in the cAMP-dependent/PKA-independent pathway of progesterone production in mature follicles of human ovaries. Additionally, an Epac-selective cAMP analog and its metabolites increase the expression of P450scc (CYP11A1) mRNA and cortisol synthesis in bovine adrenocortical cells (Enyeart and Enyeart, 2009). Despite the enhanced increase in progesterone synthesis after simultaneous treatment with CPT and Phe, the P450scc mRNA levels did not change compared with Phe treatment alone. This indicates the possibility that Epac may mediate another pathway that is independent from P450scc expression in BeWo cells. Collectively, our data suggest that the cAMP/PKA signaling pathway plays a major role in functional trophoblast differentiation (which includes hCG and progesterone synthesis), whereas the cAMP/Epac signaling pathway is involved cooperatively in the differentiation of human trophoblasts (Fig. 6).
We found that CPT mimicked the effect of the non-selective cAMP analog (Db)-induced BeWo cell fusion. In addition, the CPT- or Db-evoked syncytial fusion was retained in the presence of the PKA inhibitor. These findings suggest that the PKA-independent cAMP-Epac signaling pathway is involved in syncytialization. Furthermore, CPT- or Db-stimulated syncytialization was inhibited by the knocking down of Epac1 or Rap1, but not of Epac2. Interestingly, the PKA-selective cAMP analog (Phe) did not stimulate BeWo cell syncytialization. These results support the conclusion that the Epac1-mediated Rap1 activation is related to the cAMP-induced syncytialization of BeWo cells (Fig. 6). It has been suggested that cAMP-mediated syncytialization is PKA dependent (Keryer et al., 1998) and probably involves up-regulation of the transcription factor glial cells missing a (GCMa), which results in up-regulation of the human endogenous retrovirus (HERV)-encoded protein syncytin1 in BeWo cells (Yu et al., 2002; Chang et al., 2005; Knerr et al., 2005). Furthermore, the envelope protein of another HERV member, syncytin 2, which is expressed specifically in placental CTB, plays a crucial role in the process of FSK-induced trophoblast cell fusion (Malassine et al., 2007; Vargas et al., 2009). The synergistic action of PKA and Epac in cAMP signaling is required to differentiate mouse 3T3-L1 fibroblasts to adipocytes (Petersen et al., 2008). In contrast, persistent stimulation of PKA inhibits differentiation to adipocytes (Li et al., 2008). These results may indicate that the strict regulation of PKA and Epac in cAMP-mediated signaling is required for appropriate cellular responses. In the present study, we did not gather data pertaining to how Epac/Rap1-mediated cAMP signaling may stimulate BeWo cell syncytialization. Several reports have demonstrated that the formation of gap junctions, which enable cell-to-cell interactions, is required for trophoblast fusion (Cronier et al., 2003; Frendo et al., 2003). Connexin 43
(Cx43), which is a component of gap junctions, is up-regulated during syncytialization, and the antisense-mediated knockdown of Cx43 expression inhibits syncytialization in isolated villous CTB (Frendo et al., 2003). An Epac-selective cAMP analog stimulates gap-junction formation via the accumulation of Cx43 at cell–cell contact sites in rat cardiac myocytes (Somekawa et al., 2005), although the

**Figure 5** Effects of the knockdown of Epac1, Epac2 and Rap1 on the CPT-, Db- or FSK-induced syncytialization of BeWo cells. BeWo cells were transfected with non-targeting control (Cont), Epac1, Epac2 or Rap1 siRNAs and were then cultured with 0.5 mM CPT, 0.5 mM Db or 10 μM FSK for 48 h. (A) The expression of Epac1, Epac2 or Rap1 was determined using immunoblotting. The same blot was stripped and re-probed with anti-GAPDH antibody as a loading control. (B) Cells were immunostained with anti-desmosomal protein antibody (red) and DAPI (blue) to visualize syncytialization. Representative pictures are shown and syncytialized cells are marked with a stippled line. (C) The number of syncytialized cells in five areas selected randomly was counted. The data are presented as ratios of the control group and are shown as mean ± SEM from four independent experiments. **p < 0.01 versus Cont siRNA/CPT, ***p < 0.01 versus Cont siRNA/Db, ††p < 0.01 versus Cont siRNA/FSK.
physiological relationship between Cx43 and Epac in trophoblasts has not been examined. Furthermore, activation of Rap1 is necessary for the accumulation of Cx43 and of the cell-adhesion factor N-cadherin at cell–cell contact sites (Kooistra et al., 2007). Epac-mediated cAMP signaling may control the process of cell–cell contact via the formation of gap junctions, which are a prerequisite for syncytialization. The precise mechanisms of Epac1/Rap1-mediated fusion of trophoblasts warrant further investigation.

Further research focusing on the role of Epac in EVT migration and invasion may also be an important subject, as Epac may be associated with various types of cell migration, e.g. in melanoma (Baljinnyam et al., 2009), vascular smooth muscle cells (Yokoyama et al., 2008) and epithelial cells (Lyle et al., 2008).

In conclusion, the present study demonstrated that cAMP-mediated Epac signaling may regulate syncytialization via Rap1 and in part be involved in functional differentiation, which includes hCG and progesterone production, of human trophoblasts. This may imply that trophoblast differentiation, i.e. endocrine changes and syncytialization, are regulated differentially by cAMP signaling, although these functional and morphological aspects of differentiation occur synchronously in the process of placental formation.

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**References**


