RhoE Is Regulated by Cyclic AMP and Promotes Fusion of Human BeWo Choriocarcinoma Cells

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Abstract

Fusion of placental villous cytotrophoblasts with the overlying syncytiotrophoblast is essential for the maintenance of successful pregnancy, and disturbances in this process have been implicated in pathological conditions such as pre-eclampsia and intra-uterine growth retardation. In this study we examined the role of the Rho GTPase family member RhoE in trophoblast differentiation and fusion using the BeWo choriocarcinoma cell line, a model of villous cytotrophoblast fusion. Treatment of BeWo cells with the cell permeable cyclic AMP analogue dibutyryl cyclic AMP (dbcAMP) resulted in a strong upregulation of RhoE at 24h, coinciding with the onset of fusion. Using the protein kinase A (PKA)-specific cAMP analogue N6-phenyl-cAMP, and a specific inhibitor of PKA (14–22 amide, PKI), we found that upregulation of RhoE by cAMP was mediated through activation of PKA signalling. Silencing of RhoE expression by RNA interference resulted in a significant decrease in dbcAMP-induced fusion. However, expression of differentiation markers human chorionic gonadotrophin and placental alkaline phosphatase was unaffected by RhoE silencing. Finally, we found that RhoE upregulation by dbcAMP was significantly reduced under hypoxic conditions in which cell fusion is impaired. These results show that induction of RhoE by cAMP is mediated through PKA and promotes BeWo cell fusion but has no effect on functional differentiation, supporting evidence that these two processes may be controlled by separate or diverging pathways.

Introduction

The syncytiotrophoblast layer of the human placenta is a large multinucleated epithelium forming the outer surface of the placental villi. It is in direct contact with maternal blood and is the site of key placental functions such as nutrient and gas exchange, and the synthesis of steroid and peptide hormones [1]. The formation, growth and maintenance of the syncytiotrophoblast throughout pregnancy depends on continuous fusion with underlying mononuclear villous cytotrophoblast stem cells [2]. Disturbances in fusion may be involved in pregnancy disorders such as pre-eclampsia and intra-uterine growth retardation [3,4]; however, the mechanism by which it occurs remains poorly understood. Isolated villous cytotrophoblasts aggregate and fuse in vitro to form multinucleated syncytiotrophoblast [5] and this is enhanced by treatment with cAMP, or with agents which increase intracellular cAMP levels. A number of proteins have been implicated in the fusion process, including envelope proteins derived from human endogenous retroviruses (HERVs) [6] and their receptors [7,8], glial cells missing 1 (GCM1) [9], connexin 43 [10] and ADAM proteins [6].

The Rho family of Ras-like GTPases comprises at least 23 members of this subfamily lack intrinsic GTPase-activating proteins (GAPs) respectively [11]. Several studies have described a role for Rho GTPases in trophoblast processes such as cell migration [13] and cytokkeletal reorganization [14] but no data have been reported on any possible role in human cytotrophoblast fusion.

RhoE/Rnd3 is a member of the Rnd subfamily of Rho GTPases, which also comprises Rnd1 and Rnd2 [15]. Unlike other Rho GTPases, the members of this subfamily lack intrinsic GTPase activity, do not bind GEFs and GAPs and therefore exist predominantly in a constitutively active GTP-bound state. Hence, their activity and function within the cell is regulated by their expression level and localization. RhoE has been shown to regulate cytokkeletal reorganisation and cell motility through inhibition of RhoA activity [16], and plays a role in processes such as cell proliferation and cell cycle progression [17,18], apoptosis [19] and differentiation [20]. These various functions of RhoE appear to be cell type- and context-dependent. A possible role for RhoE in cell fusion has been demonstrated by the finding that, in myoblasts, RhoE expression increases under the onset of cell fusion, and this upregulation is required for the inhibition of RhoA and ROCK1 activities and subsequent myoblast fusion [21]. In this study we report for the first time that RhoE plays a role in human cytotrophoblast fusion using the BeWo choriocarcinoma cell line, a well characterised model which shares important properties with freshly isolated human villous cytotrophoblasts, most significantly the ability to fuse and form large multinucleated syncytiot.
show that RhoE is upregulated by cyclic AMP via activation of protein kinase A, and that knockdown of RhoE by RNA interference inhibits cell fusion. Finally, we show that RhoE upregulation is attenuated under hypoxic conditions in which cell fusion is impaired.

Materials and Methods

Ethics Statement

This study was approved by the Oxfordshire Research Ethics Committee C.

Reagents

Dibutyryl cyclic AMP (dbcAMP) was obtained from Sigma, UK. The PKA-selective cAMP analog N\(^6\)-phenyl-cAMP (Phe) was obtained from Biolog Life Science Institute, Germany. PKA inhibitor 14–22 amide (PKI) was purchased from Merck, UK.

Cell culture

BeWo cells and JEG-3 cells, both obtained from the European Collection of Cell Cultures (Porton Down, UK), were cultured in full growth medium (Dulbecco’s modified Eagle’s medium/Ham’s F12 supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 \(\mu\)g/ml streptomycin (Sigma) and 10% (v/v) fetal calf serum (Serum Laboratories International)). Cells were grown as a monolayer at a density of 10\(^7\) cells per 75 mm\(^2\) flask at 37°C in 95% air and 5% CO\(_2\), with medium changed every 48 h. For replating.

Isolation of human primary villous cytotrophoblasts

Term placentae (n = 3) were obtained with informed consent after delivery by elective caesarean section in the John Radcliffe Hospital, Oxford. Women with medical complications or previous perinatal deaths were excluded. Only singleton pregnancies without fetal abnormality or fetal growth restriction, i.e. >10th centile, and ≥37 weeks gestation were included. Villous cytotrophoblasts were prepared as described previously [23]. After digestion partially pure cytotrophoblasts were isolated using a 5–70% Percoll gradient (Amersham Biosciences UK). The fraction between densities of 1.042 g and 1.068 g was aspirated, washed with PBS and fixed in methanol at −20°C for 10 minutes. Intercellular boundaries were then visualized by immunocytochemical staining with an antibody to desmosomal protein as described above. Ten random fields containing approximately 300 nuclei each were photographed for subsequent analysis. Composite images of Hoechst 33342 stained nuclei and Alexa Fluor 488 stained desmosomal protein were made, then all nuclei were counted and the percentage of nuclei contained within multinucleate (≥2 nuclei) syncytia was calculated, as described previously [24].

Statistical analysis

The data presented represent mean ± SEM of at least three separate experiments. Differences between treatment groups were analysed by ANOVA and a P-value of <0.05 was considered to be statistically significant.

Results

RhoE expression is upregulated by cyclic AMP in BeWo cells and is associated with cell fusion

BeWo cells fuse in response to treatment with cyclic AMP [24,25]. Here we sought to determine the effect of the cell-
permeable cyclic AMP analogue dibutyryl cyclic AMP (dbcAMP) on RhoE expression in BeWo cells. Confluent cells were treated with low-serum medium in the presence or absence of dbcAMP at 1mM, a concentration which has previously been shown to induce cell fusion [24]. RhoE expression was then detected by immunoblotting. Treatment with dbcAMP significantly increased RhoE expression after 24h compared with control (Figs. 1A and B). To confirm stimulation of cell fusion by dbcAMP, intercellular boundaries were visualised by labelling desmosomal protein. Fusion was then quantified by calculating the percentage of nuclei contained within multinucleated syncyta. As expected, treatment with dbcAMP significantly increased fusion compared to control (12.5% vs 3.5% and 19.0% vs 9.6% at 24h and 48h respectively, Figs. 1C and D). We then assessed the effect of dbcAMP on RhoE expression in JEG-3, a human choriocarcinoma cell line which undergoes less fusion in response to cAMP compared to BeWo [26]. In JEG-3 cells, both RhoE upregulation (Figs. 2A and 2B) and intercellular fusion (Fig. 2C) were significantly attenuated in response to dbcAMP treatment compared to BeWo cells. Taken together, these results suggested a correlation between RhoE expression and cell fusion.

**Upregulation of RhoE by cyclic AMP is mediated via protein kinase A**

Cyclic AMP can exert its effects by protein kinase A (PKA)-dependent and -independent mechanisms [27]. To explore the role of PKA in the upregulation of RhoE by cyclic AMP we treated BeWo cells with a PKA-selective cAMP analog, N'-phenyl-cAMP (Phe), for 24h and assessed RhoE expression by immunoblotting. Treatment with Phe resulted in an induction of RhoE expression equal to that given by dbcAMP treatment (Fig. 3A). We then investigated the effect of the specific PKA inhibitor 14–22 amide (PKI) on dbcAMP-induced RhoE expression. BeWo cells were pretreated with PKI or vehicle for 1h then incubated with dbcAMP with or without PKI for 24h and assessed for RhoE expression. PKI inhibited dbcAMP upregulation of RhoE (Fig. 3B). These data show that RhoE upregulation by cAMP is mediated through activation of PKA.

**RhoE knockdown inhibits BeWo cell fusion**

Since we found that BeWo cell fusion was associated with an upregulation of RhoE, we hypothesised that RhoE may play an active role in driving this process. To test this hypothesis we used

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**Figure 1. Effect of cyclic AMP on RhoE expression and fusion in BeWo cells.** BeWo cells were treated with or without 1mM dbcAMP and studied at the indicated times. Cell lysates were made and expression of RhoE and β-actin was assessed by immunoblotting (A) and densitometric analysis of blots (B). Cells were fixed, immunostained for desmosomal protein (green) and counterstained with Hoechst 33258 (blue) (C) and cell fusion was quantified (D) as described in Materials & Methods. Results are presented as mean ± SEM for three separate experiments. *p<0.05, **p<0.01 compared with control (determined by ANOVA).

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RNA interference to knock down RhoE expression. BeWo cells were transfected with siRNA duplexes targeted to human RhoE mRNA (RhoE siRNA) or a non-silencing control which has no homology to any known mammalian gene, and then treated with dbcAMP to stimulate RhoE expression. RhoE was efficiently downregulated by transfection with RhoE siRNA, as demonstrated by an immunoblot of BeWo cell lysates following dbcAMP treatment (Fig. 4A). Levels of β-actin were unaffected by transfection with either RhoE or non-silencing siRNA. Densitometric analysis revealed that transfection with RhoE siRNA resulted in a 73% knockdown of RhoE expression after 24h dbcAMP treatment compared with the non-silencing control.

We next examined the effect of RhoE knockdown on BeWo cell fusion. Cells were transfected with RhoE or control siRNA then treated with low-serum medium containing 1mM dbcAMP. RhoE siRNA-transfected cells showed a significant decrease in the percentage of nuclei contained in syncytia compared with cells transfected with non-silencing control (7.0% vs 11.9%, and 16.6% vs 25.2%, at 24h and 48h respectively; Fig. 4B). We then determined whether RhoE downregulation affected the expression of hCG and placental alkaline phosphatase (PLAP), two markers of biochemical differentiation of BeWo cells. Expression of both these proteins was induced by dbcAMP treatment but their levels were unchanged in RhoE siRNA-transfected cells compared to non-silencing control following dbcAMP treatment, as assessed by immunoblotting (Fig. 4C). These results suggest that RhoE plays a role in BeWo cell fusion, but is dispensable for the induction of some proteins associated with biochemical differentiation.

RhoE upregulation by cyclic AMP is inhibited by hypoxia

It has long been known that fusion of cytotrophoblasts is inhibited under hypoxic conditions [28]. We proposed that, since RhoE plays a role in BeWo cell fusion, its induction by dbcAMP may be impaired under hypoxia. To test this, cells were grown to confluence and cultured at 20% O2 (normoxia) or 1% O2 (hypoxia) for 24h, then treated with 1mM dbcAMP at 20% or 1% O2 for a further 24h. RhoE expression was then assessed by immunoblotting. Although 6.5–8.6% O2 is regarded as placental normoxia [29], we used 20% O2 as normoxic conditions in these experiments as BeWo cells have been adapted in culture to grow at 20% O2 and thus this represents normoxia for these cells. Figure 5 shows that the induction of RhoE by dbcAMP at 24h was reduced in hypoxic conditions compared to normoxia. These data suggest that the inhibition of BeWo cell fusion under hypoxia may be due, at least in part, to impaired RhoE induction.

RhoE is expressed in primary human villous cytotrophoblasts

Since RhoE plays a role in BeWo cell fusion, we addressed the relevance of these observations to normal human trophoblast by...
assessing the expression of RhoE in isolated primary human villous cytotrophoblasts. We found that RhoE was strongly expressed in these cells, which fuse spontaneously in culture [5], compared with the very weak expression in untreated, non-fusing BeWo cells (Fig. 6), suggesting that RhoE may play a role in normal human trophoblast functions, including fusion.

Discussion

In this study we demonstrate for the first time that RhoE is regulated by cyclic AMP and is involved in the cyclic AMP-mediated fusion of the BeWo human choriocarcinoma cell line. These results suggest that RhoE may play an important role in the regulation of trophoblast fusion in normal and/or pathological pregnancies.

Several members of the Rho GTPase family, such as RhoA, Rac1 and cdc42, have been described in human cytotrophoblasts and shown to play a role in their migration [13]. However, a role for members of the Rho family of proteins in cytotrophoblast fusion has not been reported to date. In myoblasts, RhoE is required for intercellular fusion resulting in the formation of myotubes. In these cells RhoE expression increases until the onset of fusion before returning to its basal level once fusion is under way [21]. To date, no data exist on the expression of RhoE in primary human cytotrophoblasts or in the BeWo cell line, a well-characterised model of human cytotrophoblast differentiation and fusion [22]. Given its role in myoblast fusion, we hypothesised that RhoE may also be expressed in BeWo cells and play a role in their fusion.

Cyclic AMP treatment has long been known to promote fusion of both primary human cytotrophoblasts [30] and BeWo cells [24,25], and to upregulate several proteins involved in fusion, including syncytin-1 [31], syncytin-2 [32], MFSD2A [33] and CD9 [34]. In the present study, treatment of BeWo cells with the cell-permeable cAMP analogue dbcAMP led to an upregulation of RhoE, concomitant with an increase in cell fusion. Since RhoE lacks intrinsic GTPase activity and is not regulated by GEFs and GAPs, control of its expression level, together with post-translational modification, is an important mechanism for the regulation of its function within the cell. A number of different stimuli have been shown to induce RhoE expression via transcriptional regulation, including chemotherapeutic agents [35], ultraviolet irradiation [36] and estradiol [37]. Phosphorylation of RhoE by ROCK1 and PKC also upregulates its expression by increasing its stability through prevention of proteasomal degradation [38,39].

To our knowledge, this is the first report showing upregulation of RhoE by cAMP. Interestingly, induction of RhoE by dbcAMP
was significantly impaired in JEG-3 cells compared to BeWo cells. Although some studies have reported that JEG-3 cells are non-fusogenic [40,41], we, in this study, and others [26] have shown that they do undergo some fusion in response to cAMP but at a significantly reduced level compared to BeWo cells. These results led us to hypothesise that this correlation between cAMP-induced

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**Figure 4. Effect of RhoE knockdown on BeWo cell fusion and differentiation.** BeWo cells were transfected with 50nM RhoE siRNA or a non-silencing control then treated with 1mM dbcAMP and studied at the time points indicated. Cell lysates were made and expression of RhoE and β-actin was assessed by immunoblotting (A). Cells were also fixed and immunostained for desmosomal protein and cell fusion quantified (B) as described in Materials & Methods. Cell lysates were also studied for expression of PLAP, β-hCG and β-actin by immunoblotting (C) with densitometric analysis normalised to β-actin expression (D). Results are presented as mean ± SEM for four separate experiments. *p<0.05 compared with non-silencing control (determined by ANOVA).

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**Figure 5. Effect of hypoxia on cAMP-induced RhoE expression.** BeWo cells were cultured at 20% O₂ (normoxia) or 1% O₂ (hypoxia) for 24h, then treated with 1mM dbcAMP at 20% or 1% O₂ for a further 24h. Cell lysates were made and RhoE and β-actin expression was assessed by immunoblotting (A) with densitometric analysis of RhoE expression normalised to β-actin expression (B). A representative blot from three separate experiments is shown.

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RhoE expression and subsequent fusion may indicate a direct role for RhoE in BeWo cell fusion. In support of this hypothesis we found that knockdown of RhoE expression by RNA interference resulted in a significant inhibition of BeWo cell fusion. Although significant, the reduction in fusion we observed following RhoE depletion was relatively modest. This may be due to upregulation of other Rnd subfamily members in response to dbcAMP, or may indicate the concurrent activation of alternative, Rnd subfamily-independent pathways which also promote cell fusion. However, it may also be the result of insufficient knockdown; although immunoblot analysis revealed 73% knockdown, there was still clearly some upregulation of RhoE in response to dbcAMP which may have been sufficient to induce some fusion. As in myoblasts [21], RhoE expression is maximal at the onset of fusion and then declines at later time points despite a further increase in fusion, suggesting that RhoE is required relatively early in the fusion pathway and is no longer required once fusion is complete.

Interestingly, expression of two differentiation-associated proteins, hCG and PLAP, was unaffected by RhoE knockdown, suggesting that RhoE is required for fusion but may be dispensable for biochemical differentiation. This supports the findings of recent work which showed that treatment of BeWo cells with a PKA inhibitor, H-89, led to an inhibition of fusion but had no effect on hCG expression [42], and suggests that trophoblast fusion and functional differentiation may be controlled by separate or diverging pathways. Similarly, in myoblasts knockdown of RhoE impairs myotube formation but has no effect on the expression of myogenin and troponin T, two muscle-specific proteins [21].

Cyclic AMP can exert its effects through activation of protein kinase A, exchange protein directly activated by cAMP and cAMP-gated ion channels. Using a PKA-specific cAMP analogue and a pharmacological PKA inhibitor we found that upregulation of RhoE by cAMP in BeWo cells is mediated through the PKA pathway. Activation of PKA has been shown to promote trophoblast fusion [30]. Many effects of PKA are elicited via its phosphorylation of cyclic AMP response element binding protein (CREB) at Ser133 and subsequent transcription of target genes [27]. In BeWo cells PKA-induced CREB phosphorylation leads to increased transcription of GCM1 [43,44], a transcription factor which is essential for trophoblast fusion. Furthermore, PKA stimulates CREB-binding protein-mediated acetylation of GCM1, resulting in an elevation of its activity [45]. Therefore, it is tempting to speculate that cAMP-PKA signalling may upregulate RhoE expression through increased expression and/or activation of GCM1. This notion is supported by our finding that upregulation of RhoE by cAMP is attenuated under hypoxic conditions, since GCM1 activity is decreased in trophoblasts in hypoxia as a result of enhanced degradation [46], and leads us to speculate that this attenuation of RhoE expression may form part of the mechanism by which BeWo cell fusion is inhibited in hypoxia [47]. However, we cannot rule out the possibility that RhoE may be upregulated by direct phosphorylation by PKA, leading to increased stability in a manner similar to that elicited by PKCζ and ROCK1.

The mechanism by which RhoE regulates BeWo cell fusion remains to be determined. In myoblasts, upregulation of RhoE leads to a p190RhoGAP-mediated inhibition of RhoA and ROCK1 activities, which are required for fusion to take place. This may be mediated through M-cadherin which is upregulated and accumulates at cell-cell contact sites in a RhoE-dependent manner [21]. There are no reports documenting RhoA or ROCK1 activities during trophoblast fusion but it has been shown that fusion is inhibited in primary human cytotrophoblasts transfected with antisense oligonucleotides specific for cadherin-11 [48]. Therefore it may be that RhoE may promote fusion in BeWo cells by increasing cadherin-11 expression and/or localisation, possibly through a mechanism involving modulation of RhoA and ROCK1 activities. These are themes which will be explored in future work.

In conclusion, the present study has identified RhoE as a new target for cAMP-PKA signalling and a mediator of fusion in BeWo cells. Our finding that RhoE is strongly expressed in isolated primary human cytotrophoblasts indicates that further studies will be required to fully understand the role of RhoE in trophoblast function.

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Author Contributions

Conceived and designed the experiments: GPC EAL CWGR ILS. Designed the experiments: GPC XFG. Performed the experiments: GPC XFG. Analyzed the data: GPC. Wrote the paper: GPC. Critical revision of the paper: XFG EAL CWGR ILS.

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