CD9 regulates transcription factor GCM1 and ERVWE1 expression through the cAMP/protein kinase A signaling pathway

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Abstract

ERVWE1 (SYNCYTIN-1), a membrane protein originating from the envelope gene of human endogenous retrovirus-W (HERV-W), mediates the fusion of mononucleated cytotrophoblasts into multinucleated syncytiotrophoblast. Though ERVWE1 has been characterized since its discovery, regulatory mechanisms associated with ERVWE1 expression have not been firmly established. We hypothesized that membrane protein CD9, involved in cell–cell fusion of fertilization and myogenesis, could be involved in the regulation of *ERVWE1* gene expression. In this study, regulatory mechanisms of *ERVWE1* expression were studied using human choriocarcinoma BeWo cells. Forskolin is an activator of adenylate cyclase, which increased CD9 and *ERVWE1* expression. The increase in CD9 expression was inhibited by a protein kinase A (PKA) inhibitor, Rp-cAMPS. These results indicate that CD9 expression is regulated by the cAMP/PKA signaling pathway. Overexpression of *CD9* increased expression levels of *ERVWE1* as well as *GCM1* (hGCMa), which is a transcription factor known to activate *ERVWE1* gene transcription. However, high *ERVWE1* expression induced by *CD9* overexpression did not result in the increase in chorionic gonadotropin, beta polypeptide production. Moreover, *CD9*-induced increase in *ERVWE1* and *GCM1* expressions were inhibited by Rp-cAMPS. These results suggest that CD9 increases *GCM1* expression via the cAMP/PKA signaling pathway, resulting in the increase in *ERVWE1* expression.

Reproduction (2009) **138** 945–951

Introduction

Trophoblasts are the first differentiated cells in embryonic development and play an important role in both embryonic implantation to the uterine endometrium and placental formation (Ferretti *et al.* 2007). Human trophoblasts are classified into two cell types; cytotrophoblasts and syncytiotrophoblasts. Cytotrophoblasts are mononucleated cells that proliferate and also form anchoring villi, from which these cells invade the maternal endometrium (Aplin 1991). Cytotrophoblasts also fuse and differentiate into multinucleated syncytiotrophoblasts (Midgley *et al.* 1963). Syncytiotrophoblasts exhibit numerous functions including the transportation of nutrients and gases and the synthesis of hormones such as placental lactogen, chorionic gonadotrophin, and progesterone, all of which are indispensable for pregnancy establishment and maintenance (Benirschke & Kaufmann 2001). Therefore, these products have been used as markers of cell–cell fusion of trophoblast cells (Jameson & Hollenberg 1993).

Although the molecular mechanism for placental morphogenesis has not yet been determined, it was demonstrated that SYNCYTINs, which are the membrane proteins derived from the envelope protein of endogenous retroviruses, play a key role in the fusion of cytotrophoblasts, resulting in the formation of syncytiotrophoblasts. Human SYNCYTINs are classified into two types, SYNCYTIN-1 (now called ERVWE1) and SYNCYTIN-2, which are derived from human endogenous retrovirus-W (HERV-W) and human endogenous retrovirus-FRD (HERV-FRD), respectively (Mi *et al.* 2000, Blaise *et al.* 2003). Both types of SYNCYTINs are fusogenic (Mi *et al.* 2000, Blaise *et al.* 2003, Malassiné *et al.* 2007) and are expressed in both cytotrophoblasts and syncytiotrophoblasts (Blond *et al.* 2000, Frendo *et al.* 2003, Malassiné *et al.* 2007). The effect of SYNCYTINs on the fusion of human cytotrophoblast cells has been well characterized. However, except cAMP/protein kinase A (PKA) signaling pathway, its regulatory mechanism has not been definitively characterized. Activation of the cAMP signaling increases *ERVWE1*

gene expression (Frendo *et al.* 2003, Chen *et al.* 2008) and can induce syncytia formation in primary cytotrophoblasts and human choriocarcinoma BeWo cells (Feinman *et al.* 1986, Mi *et al.* 2000). Activation of the cAMP/PKA signaling pathway in turn increases the expression of a transcription factor, glial cell missing factor homolog 1 (GCM1, previously called hGCMa), which binds to the upstream region of the *ERVWE1* gene and then stimulates *ERVWE1* gene transcription in a cell-specific manner (Yu *et al.* 2002, Chang *et al.* 2005).

It is still unclear which molecules regulate the cAMP/PKA signaling pathway, resulting in the activation of *ERVWE1* gene transcription. The members of the tetraspanin superfamily (transmembrane four superfamily; Maecker *et al.* 1997, Berdichevski 2001s) constitute the 'tetraspanin web', which is the complex molecular network which interacts with each tetraspanin and/or other membrane proteins (Boucheix & Rubinstein 2001). CD9, a member of the tetraspanin superfamily, is required for cell-cell fusions in which fertilization takes place (Le Naour *et al.* 2000) and also in myogenesis (Tachibana & Hemler 1999). In macrophages, CD9 regulates secretions of IL10 and IL6 via the cAMP/PKA signaling pathway (Ha *et al.* 2005). We hypothesized that CD9 regulates *ERVWE1* gene expression through cAMP/PKA signaling pathway and a transcription factor GCM1 in human trophoblast cells. Therefore, the purpose of the present study was to examine whether or not the *ERVWE1* gene is upregulated by CD9 in BeWo cells, and to identify molecular mechanisms by which the *ERVWE1* gene is regulated by CD9.

Results

CD9 expression is regulated by cAMP/PKA signaling pathway

To identify a factor(s) that was upregulated following the forskolin treatment in BeWo cells, several candidate mRNAs were examined. Among them, increase in *CD9* mRNA was found, which was subjected to further studies. During the course of such examinations, the effect of forskolin on *ERVWE1* and chorionic gonadotropin, beta polypeptide (CGB) expression was examined. It was found that *ERVWE1* and CGB expression levels in BeWo cells were increased after the forskolin treatment for 24, 48, or 72 h (Fig. 1A and B). *CD9* mRNA and protein expression levels were also increased with the forskolin treatment for 24, 48, or 72 h (Fig. 2A and B). The increase in *CD9* expression induced by forskolin was inhibited by 50 μ M Rp-cAMPS (Fig. 2C). These results indicate that *CD9* could be regulated through the cAMP/PKA signaling pathway.

Figure 1 The effects of forskolin on *ERVWE1* and CGB expression. (A) Western blot analysis of *ERVWE1* and ACTB in BeWo cells treated with 50 μ M forskolin for 0, 24, 48, or 72 h. The data from three independent experiments were analyzed by ImageJ software (NIH). Expression levels of *ERVWE1* normalized by ACTB are presented as mean $\pm$ S.E.M. from three independent experiments. * P < 0.05, compared with 0 h. The inset shows the representative data of *ERVWE1* (upper lane) and ACTB (lower lane). (B) ELISA of CGB in the medium in which cultured BeWo cells were treated with 50 μ M forskolin for 0, 24, 48, or 72 h. The data are presented as mean $\pm$ S.E.M. from three independent experiments. * P < 0.05, compared with 0 h.

CD9 up-regulates the expression of ERVWE1 via cAMP/PKA signaling pathway

To delineate molecular mechanisms by which *CD9* could possibly regulate *ERVWE1* expression, its expression was evaluated following the transfection of *CD9* expression plasmid into BeWo cells. Transient transfection of pSG5-*CD9* demonstrated the increase in *CD9* expression in BeWo cells (Fig. 3A). Transient transfection of pSG5-*CD9* also increased the abundance of *ERVWE1* and *CGB* mRNAs in BeWo cells (Fig. 3B). Similar to

Figure 2 The effects of forskolin on CD9 expression. (A) Real-time PCR analysis of *CD9* mRNA in BeWo cells treated with 50 μ M forskolin for 0, 24, 48, or 72 h. Transcript levels are shown as values relative to *ACTB* mRNA. The data are presented as mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, compared with 0 h. (B) Western blot analysis of CD9 in BeWo cells treated with 50 μ M forskolin for 0, 24, 48, or 72 h. (C) Western blot analysis of CD9 in BeWo cells treated with 50 μ M forskolin with or without 50 μ M Rp-cAMPS for 48 h.

increase in transcripts, transient transfection of pSG5-CD9 increased *ERVWE1* protein, but not CGB protein (Fig. 3C and D). Next we investigated whether or not CD9 regulated the expression of *ERVWE1* mRNA and protein via the cAMP/PKA signaling pathway. Among Rp-cAMPS doses examined, the 50 μ M dose reduced the increase in *ERVWE1* mRNA to levels similar to *ERVWE1* expression without the overexpression of the pSG-CD9 construct (Fig. 4A). The 50 μ M Rp-cAMPS inhibited *ERVWE1* protein expression (Fig. 4B). These data indicate that inhibition of the cAMP/PKA signaling pathway prevented the increase in *ERVWE1* expression, which had been induced by CD9.

CD9 increases the expression of *GCM1*, which stimulates *ERVWE1* expression

It has been documented that *ERVWE1* gene expression in BeWo cells is enhanced by the transcription factor *GCM1* (Yu *et al.* 2002, Chang *et al.* 2005). To investigate whether or not CD9 could regulate *GCM1*, of which mRNA was examined following *CD9* transfection into

BeWo cells. Overexpression of pSG5-CD9 increased the abundance of *GCM1* mRNA. Similar to *ERVWE1* regulation by CD9, increase in *GCM1* mRNA was inhibited by the use of Rp-cAMPS (Fig. 5). These observations indicate that the upregulation of *GCM1* and *ERVWE1* results from the activation of the cAMP/PKA signaling by the membrane protein CD9.

Discussion

Evidence has accumulated that *ERVWE1* plays an essential role in the formation of binucleate and syncytial trophoblast cells. A factor so far identified in the regulation of *ERVWE1* gene transcription is the transcription factor *GCM1*. In this study, we present the evidence that through cAMP/PKA signaling pathway (Fig. 2), cell membrane protein CD9 could regulate *GCM1* and *ERVWE1* gene transcription (Fig. 6).

Through increases in *ERVWE1* expression, forskolin induces cell-cell fusion in BeWo cells (Mi *et al.* 2000), resulting in the increase in CGB production (Frendo *et al.* 2003; Fig. 1). This was also documented by Jameson & Hollenberg (1993) in which CGB production in

Figure 3 The expression of *ERVWE1* and CGB in BeWo cells overexpressing CD9. (A) Western blot analysis of CD9 in BeWo cells transiently transfected with pSG5 (left lane) or pSG5-CD9 (right lane). (B) Abundance of *ERVWE1* and CGB mRNA in BeWo cells transiently transfected with pSG5 (white column) or pSG5-CD9 (black column). mRNA levels are shown as values relative to *ACTB*. The data are presented as mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, compared with pSG5. (C) Western blot analysis of *ERVWE1* in BeWo cells transiently transfected with pSG5 (left lane) or pSG5-CD9 (right lane). (D) ELISA of CGB in the medium, from which BeWo cells transiently transfected with pSG5 (left column) or pSG5-CD9 (right column) were cultured.

Figure 4 ERVWE1 up-regulation by CD9 via cAMP/PKA signaling pathway. (A) Real-time PCR analysis of *ERVWE1* mRNA expression in BeWo cells transiently transfected with pSG5 (white column) or pSG5-CD9 (black columns), which had been treated with or without 1, 10, or 50 μ M Rp-cAMPS, is shown. mRNA levels are shown as values relative to *ACTB*. The data are presented as mean $\pm$ S.E.M. from three independent experiments with duplication. a, $P < 0.05$, compared with pSG5. b, $P < 0.05$, compared with pSG5-CD9. (B) Western blot analysis of ERVWE1 expression in BeWo cells. The cells were transiently transfected with pSG5 (white column) or pSG5-CD9 (black column), which had been treated with or without 1, 10, or 50 μ M Rp-cAMPS. A representative gel from three independent western blot analyses is shown.

trophoblast cells results from the cell–cell fusion. CD9 is involved in cell–cell fusion in fertilization (Le Naour *et al.* 2000) and myogenesis (Tachibana & Hemler 1999). The previous and present findings suggest that CD9 may also be involved in the regulation of cell–cell fusion and/or ERVWE1 expression. Transient transfection of pSG5-CD9 increased the expression of ERVWE1 mRNA and protein (Fig. 3B and C). In contrast to the treatment with forskolin, CD9 overexpression increased *CGB* mRNA, but not its protein (Fig. 3B and D). Another human choriocarcinoma cell line, JEG3 cells, does not

Figure 5 Upregulation of *GCM1* mRNA by CD9. The real-time PCR analyses of *GCM1* mRNA in BeWo cells transiently transfected with pSG5 (white column) or pSG5-CD9 (black column), which had been treated with or without 1, 10, or 50 μ M Rp-cAMPS, are shown. a, $P < 0.05$, compared with pSG5. b, $P < 0.05$, compared with pSG5-CD9.

Figure 6 Proposed model of ERVWE1 regulation by CD9 in trophoblast cells. CD9 activates the cAMP/PKA signaling pathway, which increases *GCM1* expression, resulting in increases in ERVWE1 expression. The cAMP/PKA signaling pathway also increases CD9 expression, which potentiates CD9-induced expression of ERVWE1.

show any cell–cell fusion regardless of degrees of ERVWE1 expression (data not shown). In addition, whether or not cell–cell fusion is induced by over-expression of SYNCYTIN-A, which is a fusogenic protein of muridae homologous to ERVWE1, is dependent on cell types (Dupressoir *et al.* 2005). These results suggest that CD9 upregulates ERVWE1 expression, but may not induce cell–cell fusion in BeWo or JEG3 cells.

It was previously shown that ERVWE1 expression is regulated by the cAMP/PKA signaling pathway (Frendo *et al.* 2003, Chen *et al.* 2008). In this study, a question of whether or not CD9 regulates ERVWE1 expression via this signaling pathway was examined. The observation in which 50 μ M of Rp-cAMPS prevented the increase in ERVWE1 expression induced by CD9 (Fig. 4A and B) suggests that CD9 activates PKA, resulting in the increase in ERVWE1 expression. However, at this point, it is unclear how CD9 activates the cAMP/PKA signaling pathway. In fact, CD9 is widely expressed on the plasma membrane of various cell types including trophoblast cells, hematopoietic cells, endothelial cells, and numerous tumor cell lines (Maecker *et al.* 1997, Berditchevski 2001, Hemler 2003). CD9 is associated with various membrane proteins including Gi alpha subunit of a heterotrimeric G protein (André *et al.* 2006, Le Naour *et al.* 2006), which inhibits adenylate cyclase activity to decrease the production of cAMP (Birnbaumer 2007). CD9 could modulate the activity of the heterotrimeric G protein to regulate the cAMP/PKA signaling pathway. It was demonstrated that *GCM1* binds to the upstream region of the *ERVWE1* gene and then stimulates *ERVWE1* gene transcription in BeWo and JEG3 cells, but not HeLa cells (Yu *et al.* 2002). In this study, transient transfection of pSG-CD9 increased the expression levels

of *GCM1* mRNA, which was inhibited by Rp-cAMPS (Fig. 5). These results indicate that CD9 may regulate *GCM1* and *ERVWE1* expression via the cAMP/PKA signaling pathway in a cell-specific manner.

ERVWE1 expression has been implicated in pathological conditions such as preeclampsia (Chen *et al.* 2006), multiple sclerosis (Antony *et al.* 2004), and malignant tumor (Strick *et al.* 2007). Hypoxic condition, which is one of the symptoms observed in preeclampsia (Redman 1991), downregulates ERVWE1 expression (Knerr *et al.* 2003, Kudo *et al.* 2003). However, molecular mechanism for the regulation of ERVWE1 expression has not been definitively characterized. In the present study, we report for the first time that the membrane protein CD9 regulates ERVWE1 expression via the cAMP/PKA/GCM1 signaling pathway. Although CD9 interacts with many proteins that mediate signaling between the molecules inside and outside of the cells, or between the molecules on the plasma membrane, it is unclear how CD9 is activated in trophoblast cells. Cytotrophoblast cells express several types of integrins, which play an important role as adhesion molecules in trophoblast cells (Aplin *et al.* 2009). Though integrins are not typical G protein-coupled proteins, a recent report indicated that integrin beta1, which is associated with CD9 in BeWo cells (Hirano *et al.* 1999), regulates intracellular cAMP signaling via the activation of G protein (Alenghat *et al.* 2009). CD9 could modulate integrin signaling, resulting in activation of cAMP/PKA/GCM1 signaling. Further experimentation is required to clarify the molecular network involved in the regulation of CD9 and ERVWE1 expression, and of binucleate and syncytial cell formation, a prerequisite for trophoblast development and placental formation.

Materials and Methods

Cells and cell treatments

BeWo cells were routinely maintained in Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Japan Bioserum, Hiroshima, Japan), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 3.7 g/l sodium bicarbonate in a humidified atmosphere at 37 °C in air with 5% CO₂.

In each experiment, 4 or 8×10^4 cells were seeded per well of 12-well or 6-well culture plate respectively. When the cells were grown for 2–3 days to 50% confluency, culture medium was changed, to which 50 µM forskolin were added (Sigma-Aldrich). After 24, 48, or 72 h culture, the total RNA, protein and culture medium were collected. In some cases, 1, 10, or 50 µM of cAMP inhibitor, Rp-cAMPS (Sigma-Aldrich), was added 3 h before the addition of forskolin and the culture continued for 48 h. Doses of forskolin and Rp-cAMPS had been predetermined prior to the initiation of experimentations.

Plasmid construction and transient transfection

Human *CD9* cDNA, a generous gift from Dr Akira Kudo (Tokyo Institute of Technology, Yokohama, Japan), was subcloned into pGEM-T easy vector (Promega). To generate an expression construct, *CD9* cDNA was then subcloned into a eukaryotic expression vector pSG5 (Stratagene, La Jolla, CA, USA), resulting in the generation of the pSG5-*CD9* expression construct. The expression vector was transfected into BeWo cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected BeWo cells with or without forskolin or Rp-cAMPS treatment were cultured for 48 h, and total RNA, protein and/or culture media were then collected.

RNA preparation and quantitative real-time PCR

Total RNAs were extracted from BeWo cells using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instruction. Five microliters of total RNA samples (250 ng/µl) were first RT to cDNA using the cDNA RT kit (Applied Biosystems, Foster City, CA, USA) and oligo (dT) primers in a 20 µl reaction volume. The cDNA reaction mixture was diluted 1:5 using Tris-EDTA buffer.

Three microliters of the diluted cDNA reaction mixture were subjected to real-time PCR amplification using 0.5 units of ExTaq HS polymerase (Takara Biomedicals, Tokyo, Japan), 1×ExTaq HS buffer, 0.5 µM of the oligonucleotide primers, SYBR green (SYBR Green I Nucleic Acid Gel stain, Takara Biomedicals) as fluorescence intercalator and Rox reference dye (Invitrogen) in a final volume of 20 µl, and PCR amplification was carried out in Real-time PCR System (7900HT, Applied Biosystems). Each primer was designed using web-based Primer3 software as follows: *ACTB* (GenBank accession number: NM_001101.3) (F; 5'-GACTACCTCATGAA-GATCCTCPCR-3', R; 5'-CGGATGTCCACGTCGCGTCA-CACTTC-3'), *ERVWE1* (GenBank accession number: NM_014590.3) (F; 5'-ATAACCCATACCTCAAACCTCACC-3', R; 5'-GGCACTAAGAATGAGAGGAAGCA-3'), *CGB* (GenBank accession number: NM_000737.2) (F; 5'-GCTCACCAG-CATCCTATC-3', R; 5'-CCTGGAACATCTCCATCCTTG-3'), *CD9* (GenBank accession number: NM_001769.2) (F; 5'-GGACGTACTCGAAACCTTCACC-3', R; 5'-GCGGATAG-CACAGACAAGA-3'), *GCM1* (GenBank accession number: NM_003643.2) (F; 5'-GATGACAAGGAACCTGACCTAACC-3', R; 5'-AAACAAGAGGCAAGGAGACCAC-3').

The thermal profile for real-time PCR was at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s. Average cycle threshold (*C_t*) values of each sample were calculated and normalized to *C_t* values for *ACTB* using the $2^{-\Delta C_t}$ method. Each run was completed using a melting curve analysis to confirm the specificity of the amplification and the absence of primer dimers (Sakurai *et al.* 2009).

SDS-PAGE and western blotting analysis

For the detection of ERVWE1 and ACTB, whole-cells were lysed using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaVO₄, 50 mM NaF, 0.1% (w/v) SDS, and 1% (v/v) Triton X-100) containing 1 mM dithiothreitol, 2 mM

phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 µg/ml pepstatin A. Protein concentration of each sample was determined using the protein assay kit II (Bio-Rad). Protein samples (10 µg) were boiled for 5 min in the SDS sample buffer, which were then applied onto each lane of 12.5% SDS-PAGE gel under reducing conditions. For the detection of a membrane protein CD9, 5×10^4 cells were lysed in 100 µl sample buffer (20 mM Tris-HCl pH 8.6, 1% (w/v) SDS, 20% (v/v) glycerol and bromophenol blue), containing 10×SDS versus ERVWE1 detection RIPA buffer. Ten microliters of lysed sample were then applied to each lane of 12.5% SDS-PAGE gel under reducing conditions.

For western blot analysis, the proteins in the gel were transferred onto nitrocellulose membranes (Immobilon, Millipore, Bedford, MA, USA). The membranes were blocked in 5% (w/v) skim milk dissolved with Tris-buffered saline containing 0.5% (v/v) Tween (TBST) at room temperature for 1 h and then incubated with a rabbit polyclonal antibody to ERVWE1 (1:10 000, H-280, Santa Cruz, CA, USA), a mouse MAB to ACTB (1:5000, F3022, Sigma-Aldrich) or a mouse MAB to CD9 (1:10 000, K41, BMA Biomedicals AG, Augst, Switzerland) at 4 °C for 12 h. After incubation, membranes were washed three times in TBST, and incubated in donkey anti-rabbit or anti-mouse IgG-conjugated with HRP (GE Healthcare Bio-Sciences AB, Björkgatan, Sweden) at room temperature for 1 h. After washing the membrane three times in TBST, the bands were identified using ECL plus western blotting detection reagents (GE Healthcare UK Ltd, Buckinghamshire, UK).

CGB measurement in culture medium

The collected medium was centrifuged at 400 g at 4 °C for 3 min to remove cellular debris. After total protein concentrations were adjusted to 1 mg/ml using the culture medium, CGB concentration of each medium was determined using the ELISA kit (Immuno-Biological Laboratories, Hamburg, Germany), which is based on the sandwich principle and specifically detects CGB according to the manufacturer's instruction.

Statistical analysis

The concentration of CGB protein, the expression level of ERVWE1 protein and CD9 mRNA after the addition of forskolin, and the expression level of ERVWE1 and GCM1 RNAs after the addition of Rp-cAMPS were analyzed using one-way ANOVA, followed by Tukey-Kramer test. The effect of transient transfection of pSG5-CD9 on the expressions of CGB protein or ERVWE1 and CGB mRNAs was analyzed by *F* test followed by Student's *t*-test. *P* value <0.05 was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research was supported by Grants-in-aid for Scientific Research (18108004) from the Japan Society for the Promotion of Science and by the Program for Promotion of Basic Research Activities for Innovative Bioscience (BRIN).

Acknowledgements

The authors thank Dr Akira Kudo, Tokyo Institute of Technology, Yokohama, Japan, for generously providing CD9 cDNA.

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Received 4 March 2009

First decision 6 April 2009

Revised manuscript received 18 June 2009

Accepted 19 August 2009