BIOLOGY OF REPRODUCTION **79,** 815–823 (2008)
Published online before print 23 July 2008.
DOI 10.1095/biolreprod.108.069765

Functional Characterization of the Human Placental Fusogenic Membrane Protein Syncytin 2[1]

Chie-Pein Chen,[3,4] Liang-Fu Chen,[5] Su-Ray Yang,[5] Chia-Yu Chen,[4] Chun-Chuan Ko,[4] Geen-Dong Chang,[5] and Hungwen Chen[2,5,6]

Division of High Risk Pregnancy[3] and Department of Medical Research,[4] Mackay Memorial Hospital, Taipei 104, Taiwan
Graduate Institute of Biochemical Sciences,[5] National Taiwan University, Taipei 106, Taiwan
Institute of Biological Chemistry,[6] Academia Sinica, Nankang, Taipei 115, Taiwan

ABSTRACT

Fusion of cytotrophoblasts into the multinucleated syncytiotrophoblast layer is essential for the development of a functional placenta. The envelope protein of a human endogenous retrovirus W (HERV-W) family member, syncytin 1, has been shown to mediate placental cell fusion. Recently, the envelope protein of another HERV family member (HERV-FRD), syncytin 2, has been identified and shown to be highly expressed in the placenta. To better understand the biology of syncytin 2, in this study we first investigated syncytin 2 gene expression in normal and preeclamptic placentas and then characterized the functions of syncytin 2. The expression of syncytin 2 gene was decreased in preeclamptic placentas and could be stimulated by the cAMP stimulant forskolin. The endoprotease furin was found to be involved in the posttranslational cleavage of syncytin 1 and 2 polypeptides into surface and transmembrane subunits. In addition, proper association of the subunits of syncytins 1 and 2 is probably required for the functional integrity of each protein, because subunit swapping of syncytins 1 and 2 failed to generate fusogenic chimeras. Finally, we demonstrated that the disulfide bridge-forming CX_2C and CX_7C motifs found in syncytins 1 and 2 are essential for their fusogenic activities, because mutations in the CX_2C motif not only abolished fusogenesis but also functioned as dominant-negative mutants. Our results suggest that syncytin 2 may function as a second fusogenic protein for placental cell fusion.

cell fusion, placenta, pregnancy, syncytin 1, syncytin 2, syncytiotrophoblast, trophoblast

INTRODUCTION

Approximately 8% of the human genome contains sequences of retroviral origin [1, 2]. Collectively, these sequence elements are named human endogenous retroviruses (HERVs), and can be further classified into several families, such as HERV-K and HERV-W, based on the priming tRNA used for reverse transcription [2, 3]. Although most HERVs are defective in replication due to accumulated mutations in their genomes, some of them harbor functional genes with important functions for the host's physiology. For example, a functional envelope protein called syncytin 1 is encoded by the *env* gene of an HERV-W family member and is expressed in placental trophoblasts [4]. Cell-cell fusion of trophoblasts is essential for formation of the multinucleated syncytiotrophoblast layer during human placental development. In fact, syncytin 1 is known to be involved in the fusion of mononuclear cytotrophoblasts into a multinucleated syncytiotrophoblast layer because antisense oligonucleotides targeting syncytin 1 block trophoblastic fusion and differentiation [5]. Interestingly, a recent antisense study has also demonstrated that a functional envelope protein of the endogenous Jaagsiekte sheep retroviruses is involved in ovine placental growth and differentiation [6]. Clinically, decreased expression and abnormal localization of syncytin 1 was found in preeclampsia, a common pregnancy disorder with poor trophoblast differentiation and vascular dysfunction in placenta [7–9]. In contrast, increased expression of syncytin 1 protein and/or mRNA was detected in brain tissues from multiple sclerosis patients, in breast cancer cell lines and specimens, and in endometrial carcinoma [10–12]. In the human endometrial carcinoma-derived cell line RL95–2, the expression of syncytin 1 was further stimulated by steroid hormones [12]. Such an increase in syncytin 1 gene expression may be associated with the transformation phenotype of anchorage independence, because knocking down the expression of syncytin 1 by RNAi reduces the colony number and size in soft agar assay [12]. On the other hand, increased expression of syncytin 1 was found to be a positive prognostic factor in breast cancer, implicating that syncytin 1-mediated fusion between cancer cells and normal endothelial cells leads to activation of tumor suppressor genes in the hybrids, and hence prevents tumor growth [13].

Additional HERV *env* genes with fully coding sequences have been identified in the human genome by genomics and bioinformatics. Among these, the *env* genes derived from the HERVs H, K, T, R, R(b), F(c)1, F(c)2, and V families were found to encode nonfusogenic proteins when transiently expressed in mammalian cells [14]. Interestingly, an *env* gene derived from the HERV-FRD family is of special interest because, like syncytin 1, it is primarily expressed in placenta and encodes a fusogenic polypeptide termed syncytin 2 [14, 15]. By analogy, two *env* genes have recently been identified in the murine genome that encode fusogenic proteins and are highly expressed in the syncytiotrophoblast-containing labyrinthine layer. Because of their similar functional properties and close phylogenetic connection, these two murine *env* genes are termed syncytins A and B [16]. We have previously characterized the functional domains of syncytin 1 and have demonstrated that the syncytin 1 polypeptide is cleaved into surface (SU) and transmembrane (TM) subunits and that an intramolecular interaction between two heptad repeat regions in

[1]Supported by grants to H.C. from the National Science Council of Taiwan (grant 96-2311-B-001-034), National Taiwan University (grant 95R0066-BM06-02), and Academia Sinica of Taiwan, and to C.-P. C. from the National Science Council of Taiwan (grant 95-2314-B-195-018) and the Mackay Memorial Hospital (MMH-E-97001).
[2]Correspondence: FAX: 011 886 2 27889759;
e-mail: hwchen@gate.sinica.edu.tw

Received: 8 April 2008.
First decision: 25 April 2008.
Accepted: 26 June 2008.

ISSN: 0006-3363. http://www.biolreprod.org

the TM subunit is required for fusion [17]. Because the functional roles of syncytin 2 in placenta remained elusive, we wished to study the biology of syncytin 2 in terms of the regulation of syncytin 2 gene expression in placenta, the clinical implications of syncytin 2 expression in preeclampsia, and the differences in the functional properties of syncytins 1 and 2 polypeptides. In this study, we demonstrated that the expression of syncytin 2 gene is stimulated by the adenylyl cyclase activator forskolin, and a decreased expression of syncytin 2 mRNA and protein is associated with preeclampsia. At the biosynthetic level, furin is required in the cleavage process of syncytin 1 and 2 polypeptides, and the cytoplasmic domain (CTM) of syncytin 2 is required for its fusogenic activity. We also performed domain swapping experiments and mutagenesis to demonstrate that proper subunit association and disulfide bridge formation are required for the functional integrity of syncytin 1 and 2. Our results provided a better understanding of the regulation of syncytin 2 gene expression and the structure-function relationship of syncytin 2.

MATERIALS AND METHODS

Plasmid Constructs and Preparation of Syncytin 2 Antiserum

Syncytin 1 and 2 cDNA fragments were subcloned into the pTM1 plasmid (kindly provided by Dr. Bernard Moss, National Institutes of Health) to generate pTM1syn1 and pTM1syn2, respectively. Because the antisera raised against syncytin 1 and 2 do not recognize the TM subunits of syncytins 1 and 2, to facilitate the detection of TM subunit, an HA or Flag tag was attached to the carboxyl termini of syncytins 1 and 2 in the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) to generate pSyn1-Flag, pSyn1-HA, pSyn2-Flag, and pSyn2-HA. Attachment of a Flag or HA tag did not significantly affect the fusogenic activities of syncytins 1 and 2 (see Supplemental Fig. S1 available online at www.biolreprod.org). Two-step PCRs were performed to swap the TM subunit of syncytins 1 and 2, resulting in the chimeric pSU1-TM2-Flag and pSU2-TM1-Flag expression plasmids. Similarly, the chimeric pSU1-CTM2-Flag and pSU2-CTM1-Flag expression plasmids were generated by swapping the CTM subunit of syncytins 1 and 2. The syncytin 2 CTM deletion mutants pSyn2Del529-Flag, pSyn2Del520-Flag, pSyn2Del515-Flag, and pSyn2Del511-Flag were generated harboring a truncated CTM at the amino acids 529, 520, 515, and 511 of syncytin 2, respectively. Two-step PCRs were also performed to construct the CX_2C mutants pSyn1C186A-HA, pSyn1C189A-HA, pSyn1CCAA-HA, pSyn2C43A-HA, pSyn2C46A-HA, and pSyn2CCAA-HA harboring a single or double cysteine-to-alanine (C-to-A) mutation in cysteine 186 and/or 189 for syncytin 1 and cysteine 43 and/or 46 for syncytin 2. Human *FURIN* cDNA fragment was cloned into pCDNA3.1 plasmid to generate pFurin. All constructs were verified by DNA sequencing using the dideoxy chain-termination method. The cDNA fragment encoding the SU subunit of syncytin 2 (amino acids 16–350) was subcloned into pMAL-c2 plasmid (NEB, Ipswich, MA) and expressed in *Escherichia coli* according to the manufacturer's instructions. The purified recombinant protein was used to raise syncytin 2 antiserum in guinea pigs.

Cell Culture and Transfection

The 293T, BeWo, JEG-3, and LoVo cells used in this study were obtained from the American Type Culture Collection (Manassas, VA). The 293T and JEG-3 cells were maintained at 37°C in minimal essential medium-alpha medium, 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin. BeWo and LoVo cells were maintained at 37°C in Kaighn modification of Ham F-12 medium supplemented with 15% fetal bovine serum and the same antibiotics mentioned above. For transient expression, 293T and LoVo cells in six-well culture plates were infected with vTF7–3, a recombinant vaccinia virus expressing T7 RNA polymerase, at a multiplicity of infection of 10 viral particles per cell for 90 min. After infection, 293T and LoVo cells were transfected with the indicated expression plasmid(s) using the calcium phosphate coprecipitation method and Lipofectamine 2000 (Invitrogen), respectively.

Fusion Assay

Syncytin 1- and 2-mediated cell-cell fusion was monitored 12 h after transfection by light microscopy and scored as fusion events with the formation of multinucleated syncytia. Photographs were taken with an Olympus microscope (Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP50). Images were prepared for presentation using Adobe Photoshop 6.0. The fusogenic activity of syncytins 1 and 2 was calculated as a fusion index of $(N - S)/T$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted, to measure the number of fusion events [18].

Patients and Tissue

Placental tissues from different gestational ages (first trimester, $n = 11$; second trimester, $n = 8$; third trimester, $n = 21$) were obtained following elective termination (gestational age ranging from 5 to 19 wk), spontaneous preterm labor (20–36 wk) in the absence of signs and symptoms of chorioamnionitis, or normal term delivery (37–40 wk). In addition, 17 preeclamptic and 17 control placentas (27–40 wk) with matched gestational age were obtained after delivery at Mackay Memorial Hospital (Taipei, Taiwan). Each placental specimen was obtained near the umbilical cord insertion by taking a 2-cm^2 full-thickness biopsy. The conduct of this study was approved by the Ethics Committee of Mackay Memorial Hospital, and informed consent was obtained from all participating women. The diagnosis of preeclampsia was defined as patients with gestational blood pressure elevation and proteinuria occurring after 20 wk of gestation [19].

RNA Isolation, Real-Time PCR, and Ribonuclease Protection Assay

Placental tissues were homogenized in TRIZOL reagent (Invitrogen) for RNA extraction. Total RNA content was evaluated by A_{260} measurement and its integrity checked by 1% agarose gel electrophoresis. All degraded RNA samples were discarded. Total RNA (2 μg) was used for first-strand cDNA preparation using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time PCR was performed in an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) using a SYBR Green PCR Master Mix reagent kit (Applied Biosystems). The 18S rRNA was used as an internal control because rRNA constitutes the majority of cellular RNA and its level was less likely to vary in amount under different physiological conditions. The primer sequences for gene amplification, designed using Primer Express (Applied Biosystems), were AGCCTTAACGACCATGCAAGA and CTGTGCTGCCGTTAACATGTCTA for syncytin 2 and CGAGCCGCCTGGATACC and CCTCAGTTCCGAAAACCAACAA for 18S rRNA. Relative quantitation of target gene expression was calculated by the comparative C_t method, which normalizes the copy number of a target gene to that of an endogenous reference such as 18S rRNA. A validation experiment was performed to determine the relationship between log input amount of total RNA and ΔC_t. Normalized syncytin 2 gene expression relative to the reference 18S rRNA is given as previously described [9]. The ΔC_t of different groups of tissue or cells was normalized to the group of controls and expressed as a $2^{-\Delta\Delta Ct}$ value. For ribonuclease protection assay, the cDNA fragments from nucleotides 1 to 447 of the syncytin 1 and 601 to 1028 of the syncytin 2 open reading frames were subcloned for use as the riboprobe templates. Preparation of syncytin 1 and 2 riboprobes was performed using the Riboprobe System (Promega) according to the manufacturer's instructions. Ribonuclease protection assays were performed as previously described [20].

Immunoprecipitation and Immunoblotting

To detect syncytin 2 and cytokeratin 7 in placental tissues, placental extracts were prepared as previously described [9], followed by immunoblotting with syncytin 2 antiserum and a mouse anti-human cytokeratin 7 antibody, respectively. To analyze the biosynthesis of wild-type and mutant syncytin 1 and 2, cells expressing HA- or Flag-tagged syncytin 1 or 2 were harvested in RIPA (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; and 0.1% SDS) for immunoprecipitation with a mouse anti-HA or -FLAG monoclonal antibody (Sigma, St. Louis, MO). The immune complexes were then analyzed by immunoblotting with the HA or FLAG antibody.

Immunohistochemistry

Placental tissue samples were collected and snap frozen in liquid nitrogen for cryosectioning. Indirect immunostaining was performed on 5-μm-thick cryosections. The sections were air-dried and fixed in ice-cold acetone for 10 min, then rehydrated with PBS for 5 min, blocked with protein block (Dako, High Wycombe, UK) for 20 min, and incubated with the antibody against human syncytin 2 (1:100 in PBS) for 1 h at room temperature. After three washes in PBS (5 min for each wash), the tissue was incubated with the

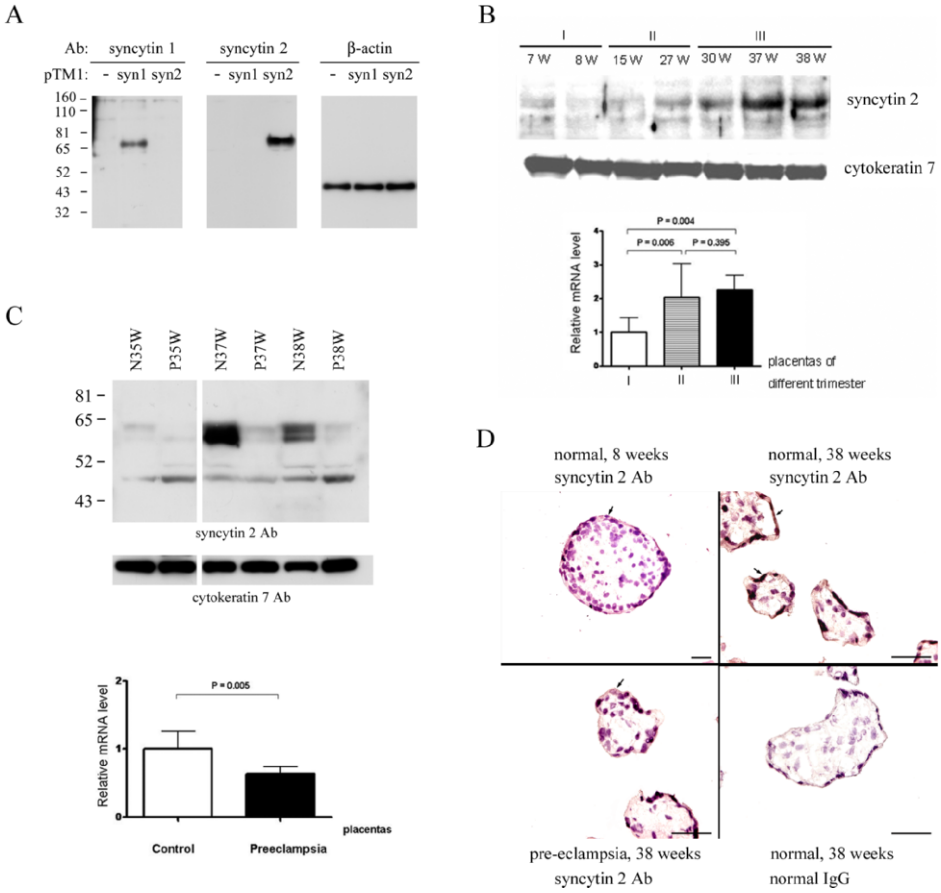

FIG. 1. Expression of syncytin 2 in normal and preeclamptic placentas. **A**) Characterization of syncytin 2 antibody. The 293T cells were infected with vTF7–3 and then transfected with 2 μg pTM1syn1 or pTM1syn2. At 24 h after transfection, cells were harvested for immunoblotting using syncytin 1, syncytin 2, and β-actin antibodies, respectively. Numbers to the left of the gels are in kDa. **B**) Expression of syncytin 2 in different gestation periods. The protein levels of syncytin 2 in placentas of different gestational periods were analyzed by immunoblotting with syncytin 2 or cytokeratin 7 antibody (upper panel). The transcript levels of syncytin 2 in placentas of different gestational periods were quantified by real-time PCR (lower panel) as described in *Materials and Methods*. The expression of syncytin 2 protein and mRNA increased as the gestational age advanced. Mean values and SDs obtained from three independent experiments are provided. W, weeks; P, *P* value **C**) Decreased expression of syncytin 2 in preeclamptic placentas. Normal (N) and preeclamptic (P) placentas from 35, 37, and 38 wk of gestation were analyzed for protein and transcript levels of syncytin 2. Mean values and SDs are provided. Numbers to the left of the gels are in kDa. **D**) Immunohistochemistry of syncytin 2. First-trimester (8 wk) and third-trimester (38 wk) placental sections of normal and preeclamptic placentas were stained with normal IgG or syncytin 2 antibody (Ab). The arrows indicate the positive staining of syncytin 2 on the syncytiotrophoblastic layers of placental villi. Bar = 60 μm.

horseradish peroxidase-conjugated secondary antibody (Dako) for 1 h at room temperature, followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. The tissues were counterstained with Mayer hematoxylin. Control experiments were carried out in which the primary antibody or secondary antibody was omitted or in which irrelevant primary antibodies were included. For BeWo cell staining, the cells were seeded on cover slips at a density of 5×10^4 cells for 24 to 48 h, the cells were then washed twice with PBS and fixed with methanol at room temperature for 30 min. Immunostaining was then performed as described above.

Statistics

Results are presented as mean ± SD. Average C_t values for each sample were calculated, and samples were grouped by gestational age. The significance of intergroup differences were assessed by one of the paired *t*, independent-samples *t*, or Mann-Whitney *U* tests, where appropriate. A *P* value of less than 0.05 was considered significant.

RESULTS

Expression of Syncytin 2 in Normal and Preeclamptic Placentas

Syncytin 2 antiserum was prepared and its specificity against syncytin 2 protein was tested by immunoblotting analyses of 293T cells transiently expressing syncytin 1 or 2. For comparison, a previously prepared syncytin 1 antiserum was used as a control [17]. As shown in Figure 1A, the syncytin 2 antiserum recognized the syncytin 2 protein but not the syncytin 1 protein and, vice versa, the syncytin 1 antibody only recognized the syncytin 1 protein. To investigate syncytin 2 expression in placenta, we analyzed the syncytin 2 protein and transcript levels in placentas of different gestation periods by immunoblotting and quantitative RT-PCR analyses, respectively. An increasing level of syncytin 2 protein was detected in placentas from the first to the third trimester (Fig. 1B, upper panel). Correspondingly, a significant increase in the level of syncytin 2 transcript was observed in the second and third trimesters compared with the first trimester (Fig. 1B, lower panel). However, there was no significant difference in the syncytin 2 transcript level between the second and third trimesters.

We further investigated whether expression of syncytin 2 differs between preeclamptic and gestation period-matched normal placentas. As shown in Figure 1C, upper panel, the syncytin 2 protein level was lower in preeclamptic placentas than normal placentas of 35, 37, and 38 wk of gestation. Correspondingly, the syncytin 2 transcript level was also significantly lower in preeclamptic placentas (n = 17) than normal placentas (n = 17; Fig. 1C, lower panel). Immunohistochemistry studies on syncytin 2 were also performed on normal placentas of 8 wk (first trimester) and 38 wk (third trimester) of gestation and preeclamptic placentas of 38 wk of gestation. The expression of syncytin 2 was localized at the syncytiotrophoblasts of placental villi of first trimester (Fig. 1D). Moreover, increased syncytin 2 expression at the syncytiotrophoblast layer was detected in the placental villi of third trimester (Fig. 1D). In contrast, the expression of syncytin 2 was significantly decreased in the third-trimester preeclamptic placenta (Fig. 1D). Taken together, these results suggest that expression of syncytin 2 levels increases significantly as gestation proceeds to the second and third trimesters but decreases in preeclamptic placentas.

Regulation of Syncytin 2 Gene Expression

Because forskolin is known to promote trophoblast cell fusion [21], we next investigated whether forskolin can

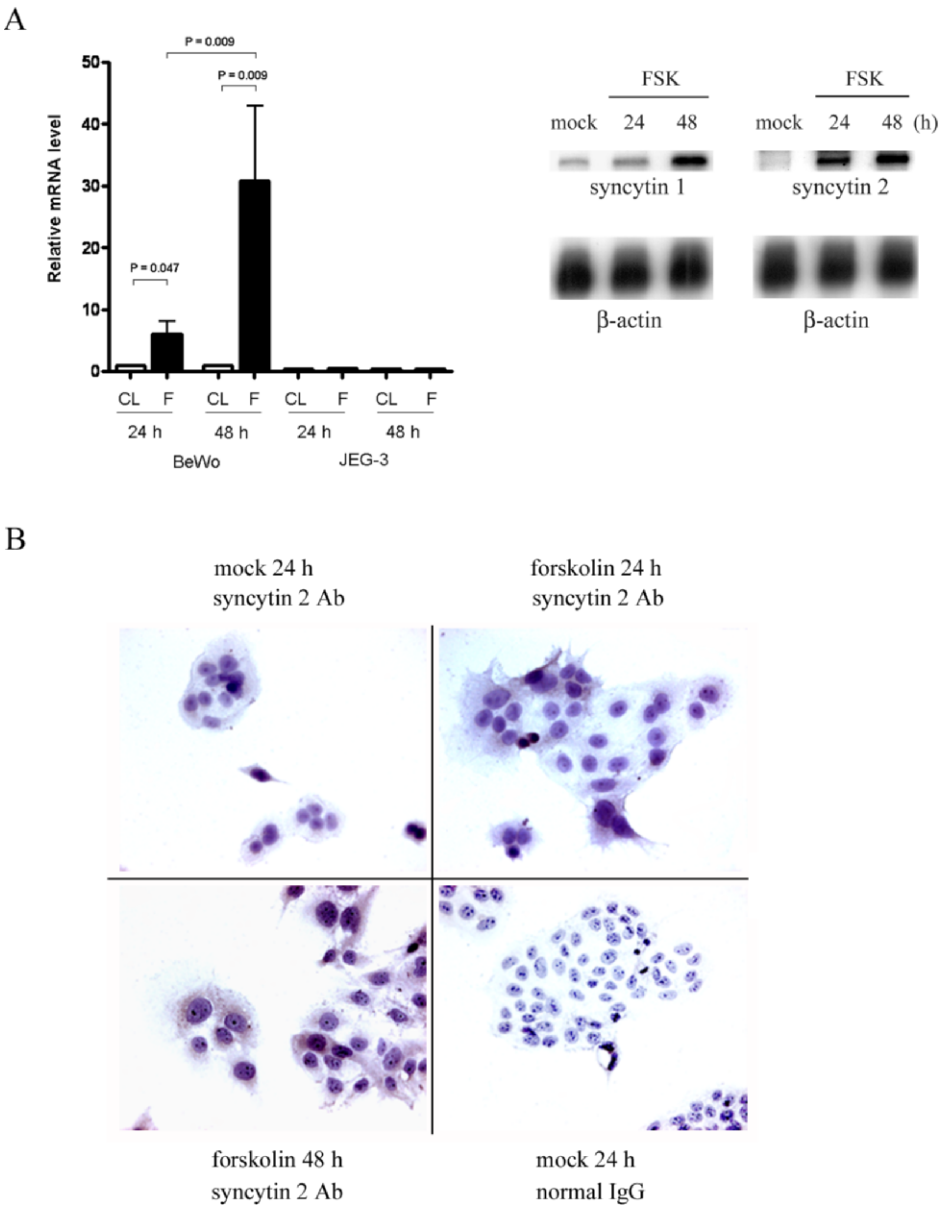

FIG. 2. Regulation of syncytin 2 gene expression. **A**) Forskolin stimulates syncytin 2 gene expression. BeWo and JEG-3 cells were mock treated (CL) or treated with forskolin (F) for 24 and 48 h, followed by real-time PCR analysis for syncytin 2 transcripts (left panel). The syncytin 1 and 2 transcripts in the mock- and forskolin-treated BeWo cells were further analyzed by ribonuclease protection assays (right panel). **B**) In a separate experiment, syncytin 2 proteins in mock- or forskolin-treated BeWo cells were analyzed by immunohistochemistry under conditions similar to those in Figure 1D (original magnification ×200). Ab, antibody.

regulate expression of the syncytin 2 gene in the human trophoblast cell line BeWo. To this end, BeWo cells were mock treated or treated with forskolin for 24 and 48 h, followed by quantitative RT-PCR. As shown in the left panel of Figure 2A, the level of syncytin 2 transcript was significantly increased by the presence of forskolin in a time-dependent manner. As a complementary approach, similar experiments were performed by ribonuclease protection assay. Interestingly, the syncytin 1 transcript could be detected in mock-treated BeWo cells, whereas the syncytin 2 transcript was barely detected (Fig. 2A, right panel). The levels of syncytin 1 and 2 transcript were significantly higher after treatment of forskolin for 24 h and further increased for 48 h (Fig. 2A, right panel). When quantitative RT-PCR was performed to study syncytin 2 expression in JEG3 cells, which are not active in cell fusion, the transcript level of syncytin 2 was barely detected and was not responsive to forskolin (Fig. 2A, left panel). Immunohistochemistry also revealed that the expression of syncytin 2 protein increased in BeWo cells treated with forskolin (Fig. 2B). Taken together, these results suggest that syncytin 2 gene expression is regulated by the cAMP signaling pathway.

Furin Is Involved in Syncytin 1- and Syncytin 2-Mediated Cell Fusion

Although both syncytin proteins are proteolytically processed into SU and TM subunits, it is not known whether the predicted endoprotease furin is involved in this process. To verify the role of furin in syncytin 1 and 2 processing, we transiently expressed syncytins 1 and 2 in 293T cells, followed by treatment with or without different amounts of furin inhibitor I. As shown in Figure 3A, cell-cell fusion mediated by either protein proceeded normally in the mock-treated cells but was inhibited in the furin inhibitor I-treated cells in a dose-dependent manner. We further found that the observed inhibitory effects of furin inhibitor I on syncytin 1- and syncytin 2-mediated cell fusion were due to inhibition of proteolytic processing of syncytin 1 and 2 polypeptides (Fig. 3B). As a complementary approach, the proteolytic processing of syncytin 1 and 2 polypeptides was tested in the furin-defective human colon carcinoma LoVo cell line (Fig. 3C) [22, 23]. When LoVo cells were transfected with pSyn1-Flag or pSyn2-Flag, the processed TM subunits of syncytins 1 and 2

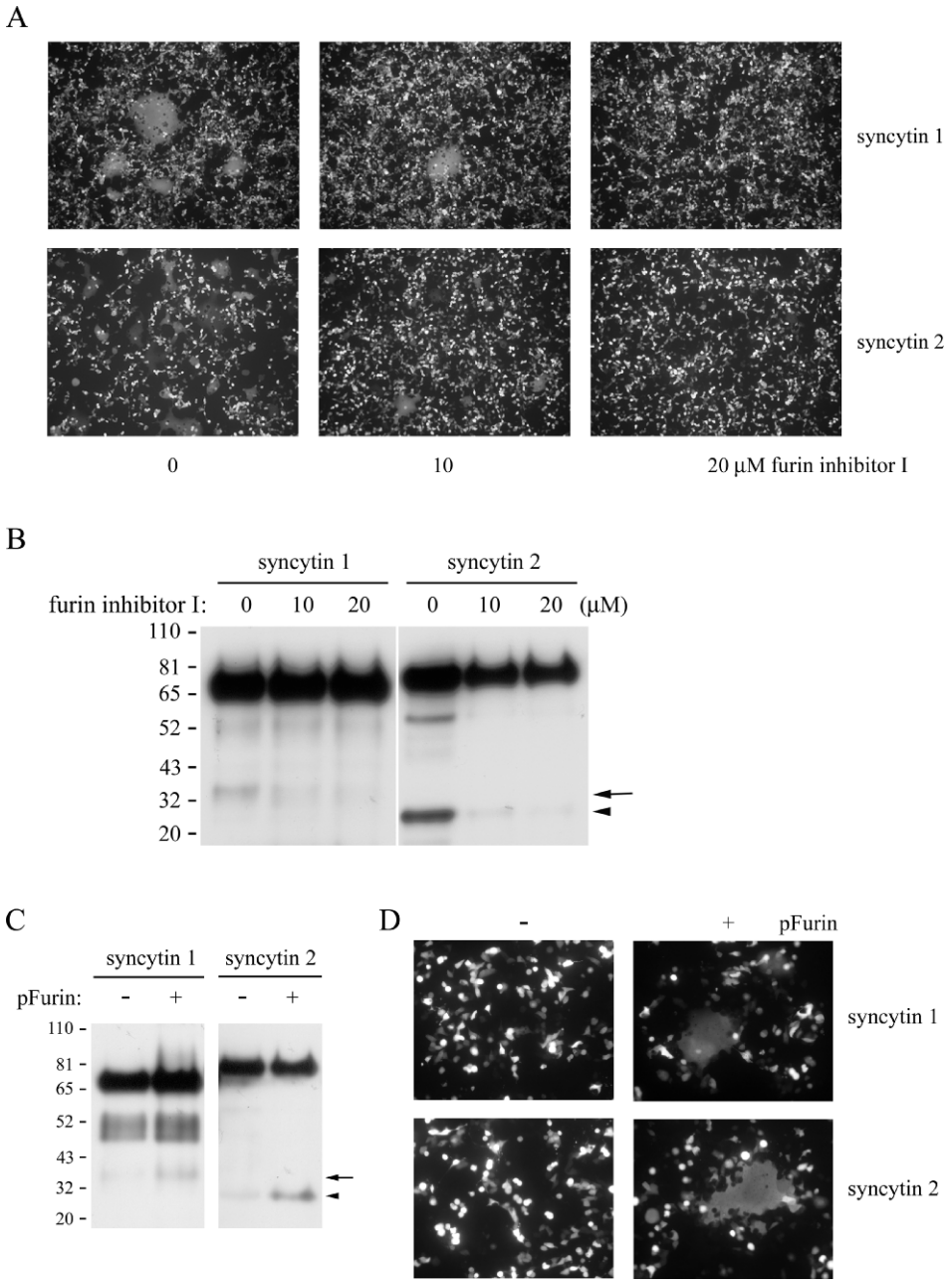

FIG. 3. Proteolytic cleavage of syncytins 1 and 2 by furin. **A**) Inhibition of syncytin 1- and syncytin 2-mediated cell fusion by furin inhibitor I. The 293T cells were transfected with 1 μg pEGFP and 5 μg pSyn1-Flag or pSyn2-Flag and then treated with or without the indicated amount of furin inhibitor I for 24 h. Cell-cell fusion was monitored by microscopy at ×40 magnification. **B**) Furin inhibitor I inhibits proteolytic cleavage of syncytins 1 and 2. In a separate experimental set similar to **A**, the processing of syncytin 1 and 2 proteins was analyzed by sequential immunoprecipitation and immunoblotting with FLAG antibody. **C**) Furin mediates the proteolytic cleavage of syncytin 1 and 2 polypeptides. LoVo cells were transfected with the indicated combination of 5 μg pSyn1-Flag and pSyn2-Flag and 2 μg pFurin, followed by sequential immunoprecipitation and immunoblotting with FLAG antibody. **D**) LoVo cells were transfected with 1 μg pEGFP and the same combination of expression plasmids as described in **C**, followed by microsopic examination at ×200 magnification. Arrow and arrowhead in **B** and **C** indicate the TM subunit of syncytins 1 and 2, respectively. Numbers to the left of the gels are in kDa.

were barely detected. However, when pFurin was cotransfected with pSyn1-Flag or pSyn2-Flag, the levels of processed TM subunits of syncytins 1 and 2 were significantly higher (Fig. 3C). Correspondingly, fusion of LoVo cells mediated by syncytins 1 and 2 was observed when pFurin was coexpressed (Fig. 3D). These results suggest that furin plays a major role in the proteolytic cleavage of syncytin 1 and 2 polypeptides.

Characterization of Syncytin 2-Mediated Cell Fusion

Our previous study demonstrated that the CTM of syncytin 1 regulates its fusogenic activity [17]. To test whether the CTM of syncytin 2 also regulates its fusogenic activity, we generated several syncytin 2 mutants with different CTM truncations for fusion assays (Fig. 4A). We quantified the fusogenic activity by analysis of a fusion index, which measures the size and number of syncytia formed. Compared with the wild type, the mutant Syn2Del529, with deletion of the amino acids 530 to 538, had a lower fusion index, which was further decreased in Syn2Del520 with a further N-terminal deletion (Fig. 4B). When the CTM was almost fully deleted, as in Syn2Del515 and Syn2Del511, we observed an approximate 90% decrease in the size and number of syncytia formed, as shown by a dramatic decrease in the fusion index in Figure 4B. Because the level of processed TM was comparable in wild-type and mutant syncytin 2 proteins (Fig. 4B), these results suggest that the CTM of syncytin 2 regulates and is required for its fusogenic activity. Because both syncytins 1 and 2 have a similar domain structure, to test whether their subunits are exchangeable, we swapped the SU subunits of syncytins 1 and 2 to create chimeric proteins (denoted as SU2-TM1 and SU1-TM2 for the proteins expressed via the expression plasmids pSU2-TM1-Flag and pSU1-TM2-Flag, respectively) for fusion assays. We could detect the processing of SU1-TM2, but not SU2-TM1 (Fig. 4C, left panel); however, cell-cell fusion mediated by either chimera was not detected (data not shown). When the CTMs of syncytins 1 and 2 were exchanged, as in SU1-CTM2

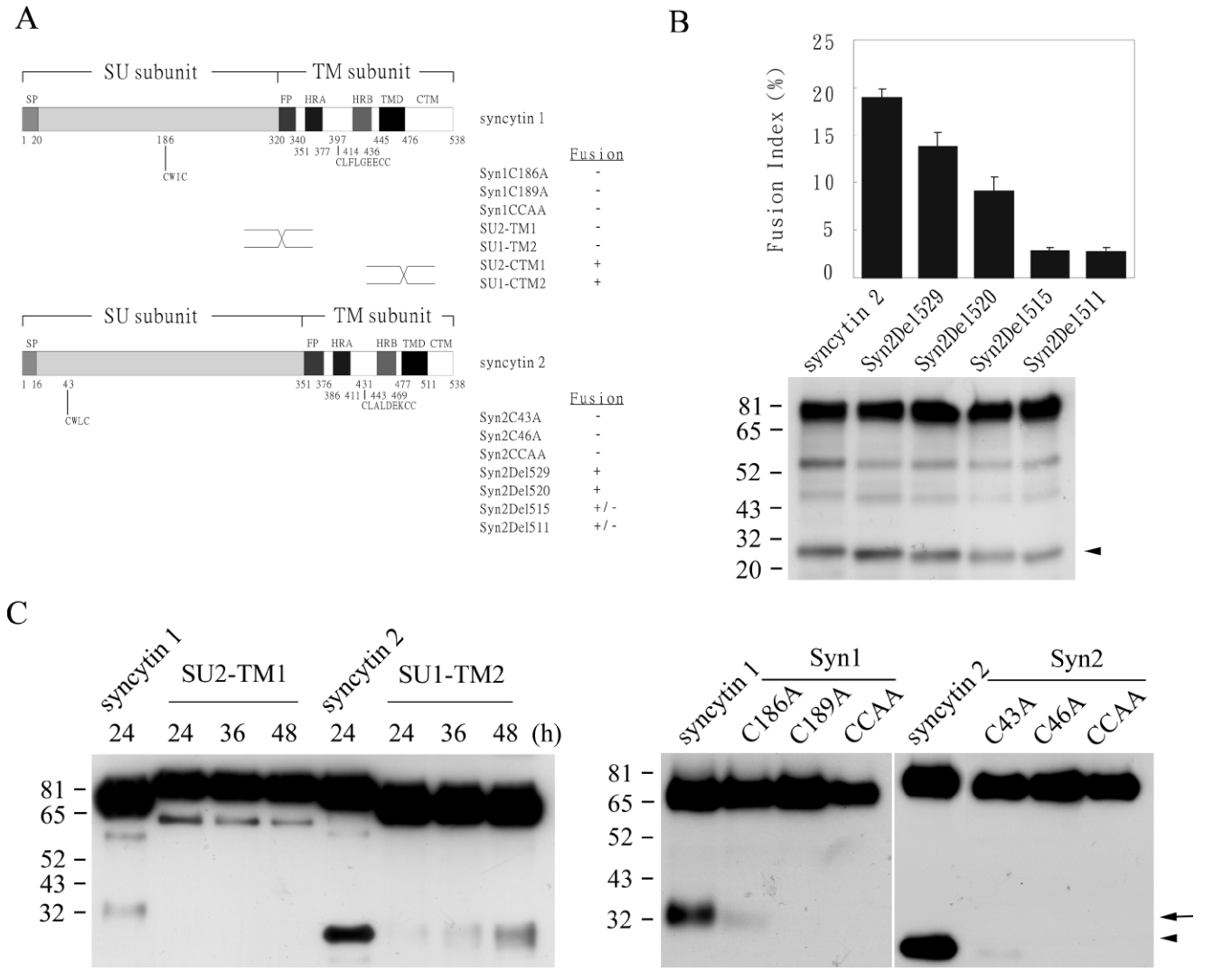

FIG. 4. Characterization of syncytin 2-mediated cell fusion. **A**) Schematic representation of syncytin 1 and 2 polypeptides. The sequences of the CX_2C and CX_7C motifs in syncytins 1 and 2 are listed. Domain swapping between syncytins 1 and 2 is indicated by a cross. In the Fusion column, + indicates the degree of cell-cell fusion mediated by mutant syncytin 1 or 2 was comparable to the wild-type; – indicates no cell-cell fusion detected; and +/– indicates the degree of cell-cell fusion was significantly reduced. **B**) The CTM of syncytin 2 regulates its fusogenic activity. The 293T cells were transfected with 1 μg pEGFP and 5 μg pSyn2-Flag or its CTM deletion mutants for 24 h, followed by fusion index analysis and sequential immunoprecipitation and immunoblotting with FLAG antibody. Values are means and SDs obtained from three independent experiments. Arrowhead indicates the TM subunit of wild-type and mutant syncytin 2. **C**) Biosynthesis of the syncytin 1 and 2 chimeric proteins and the CX_2C mutant syncytin 1 and 2 proteins. The 293T cells were transfected with 5 μg of the indicated expression plasmid, followed by sequential immunoprecipitation and immunoblotting with FLAG (for chimeric proteins) or HA (for CX_2C mutant proteins) antibody. Arrow and arrowhead indicate the TM subunit of syncytins 1 and 2, respectively. Numbers to the left of the gels are in kDa.

and SU2-CTM1, both exhibited normal cell-cell fusion (data not shown). These results suggest that the proper association between the SU and TM subunits of syncytin proteins is essential for their fusogenic activity.

A CX_2C motif in the SU and a CX_7C motif in the TM of retroviral envelopes are believed to form intersubunit and intrasubunit disulfide bridges during fusion [24, 25]. Inspection of both syncytin 1 and 2 protein sequences revealed CWTC and CLFLGEECC motifs for syncytin 1 and CWLC and CLALDEKCC motifs for syncytin 2. To characterize the functional role of these motifs in syncytins 1 and 2, we generated mutants with a C-to-A mutation in one or both of the two cysteines in the CX_2C motif, such as Syn1C186A, Syn1C189A, and Syn1CCAA for sycnytin 1, and Syn2C43A, Syn2C46A, and Syn2CCAA for syncytin 2. Interestingly, none of these mutants was fusogenic (data not shown), as only a low level of TM from Syn1C186A and Syn2C43A was detected (Fig. 4C, right panel). We speculated that if the cysteines in the CX_2C motif of syncytins 1 and 2 were involved in proper association between subunits by forming disulfide bonds, then the C-to-A mutants should impose a dominant-negative effect on the biosynthesis of wild type. To verify this speculation, we cotransfected pSyn1-Flag with pSyn1C186A-HA, pSyn1-C189A-HA, or pSyn1CCAA-HA into 293T cells for fusion assays and analyzed the level of TM-Flag (Fig. 5A, right panel). All three syncytin 1 C-to-A mutants were able to block the biosynthesis of syncytin 1-Flag, with the Syn1C186A mutant the most effective. Similarly, the Syn2C43A mutant was the most effective in blocking the biosynthesis of syncytin 2-Flag (Fig. 5A, right panel). These biochemical results further support the fusion assays in that Syn1C186A and Syn2C43A efficiently inhibited syncytin 1- and syncytin 2-mediated cell fusion, respectively (Fig. 5B). Interestingly, the syncytin 1 C-to-A mutants had no effect on syncytin 2-mediated cell fusion,

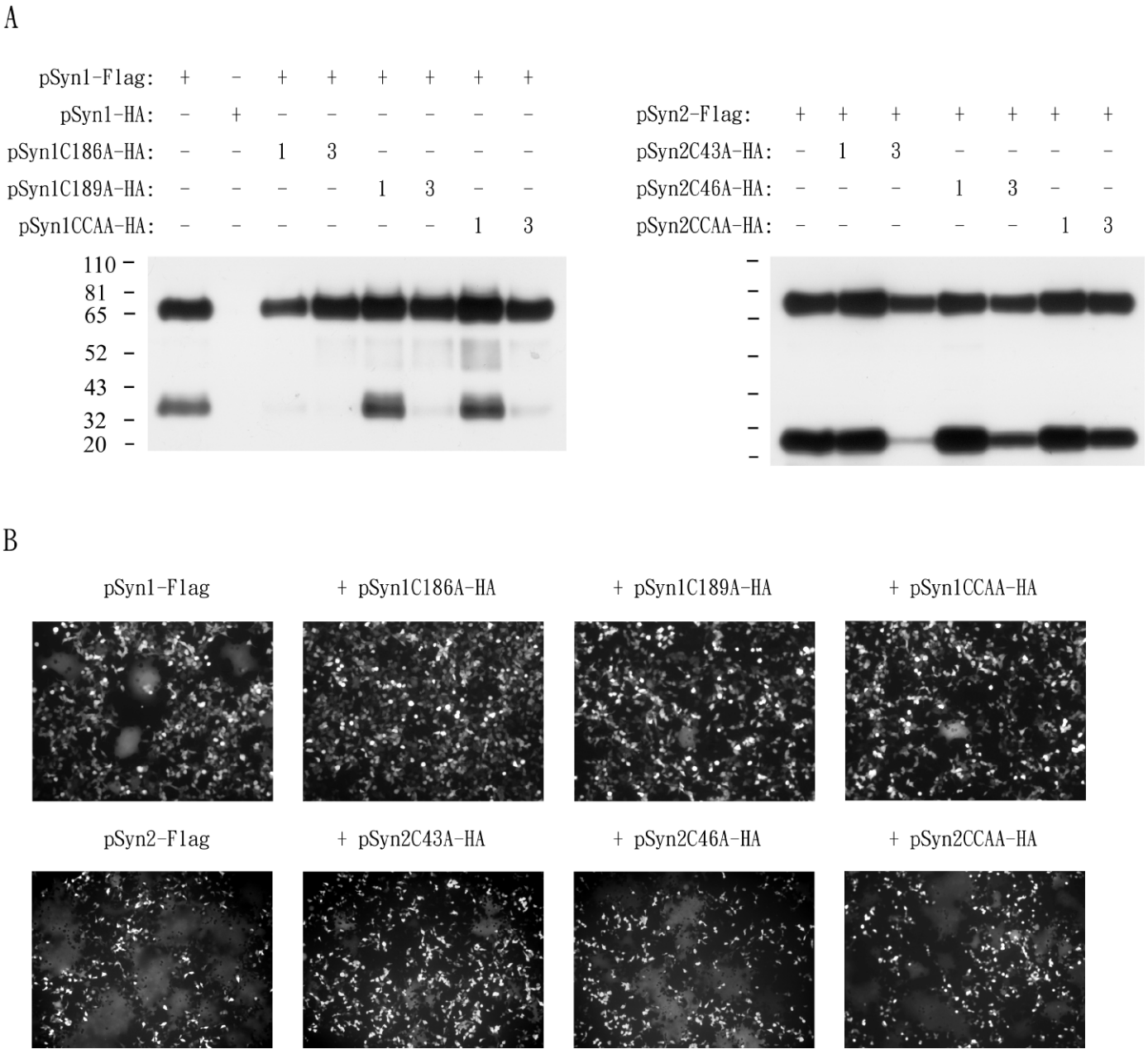

FIG. 5. Mutation of the CX_2C motif exerts a dominant negative effect on syncytins 1 and 2. **A**) The CX_2C mutant syncytins 1 and 2 interfere with the biosynthesis of wild-type syncytin 1 and 2 proteins, respectively. The 293T cells were transfected with 1 μg pEGFP and the indicated combination of wild-type or mutant syncytin 1 and 2 expression plasmids (2 μg pSyn1-Flag or pSyn2-Flag, 1 or 3 μg CX_2C mutant pSyn1-HA or pSyn2-HA). At 24 h after transfection, cells were harvested for sequential immunoprecipitation and immunoblotting with FLAG antibody. Numbers to the left of the gels are in kDa. **B**) Inhibition of the syncytin 1- and syncytin 2-mediated cell-cell fusion by the CX_2C mutant syncytin 1 and 2 proteins, respectively. The 293T cells were transfected with the expression plasmids as described in **A** for 24 h, followed by microscopic examination at ×100 magnification.

nor did the syncytin 2 C-to-A mutants have an effect on syncytin 1-mediated cell fusion (data not shown). Taken together, these results suggest that the CX_2C and CX_7C motifs in syncytins 1 and 2 are very likely involved in the formation of intersubunit and intrasubunit disulfide bridges, which are essential for their fusogenic activities.

DISCUSSION

The multinucleated syncytiotrophoblast layer on human placental villi is essential for most of the transport and endocrine activities of the placenta during pregnancy. Syncytin 1 is the first HERV envelope protein implicated in mediation of trophoblastic fusion [4]. In the present study, we investigated the expression and protein functions of syncytin 2, which is a potential second HERV envelope protein recently identified in the placenta [14]. We demonstrated that the protein and transcript levels of syncytin 2 are decreased in preeclamptic placentas and that the cAMP-elevating agent forskolin is able to positively stimulate syncytin 2 gene expression in placental cells. Similar observations have been found for syncytin 1 [4, 7, 8]. Because preeclampsia is featured with poor placental morphogenesis, and increasing the intracellular level of cAMP stimulates placental cell fusion, it is highly possible that, like syncytin 1, syncytin 2 is also a functional fusogenic protein for trophoblastic fusion. Recent immunohistochemistry and in situ

hybridization studies have also demonstrated syncytin 2 expression in placental trophoblasts, with some cytotrophoblasts containing higher levels of syncytin 2 protein and transcript [26, 27]. In addition, a significant decrease in the transcript level of syncytin 2 was detected in placentas of pregnancy-induced hypertension including patients of preeclampsia, superimposed preeclampsia, and gestational hypertension [26]. Although both syncytins 1 and 2 are fusogenic proteins in nature, a recent study has demonstrated a different physiological function specific for syncytin 2. Mangeney et al. [28] demonstrated that syncytin 2 but not syncytin 1 possesses an immunosuppressive activity when expressed in tumor cells and then transplanted to allogeneic host mice. Apparently, future investigations are warranted to understand how the fusogenic and immunosuppressive activities of syncytin 2 are regulated and coordinated in order to maintain placental function.

Although both syncytin 1 and 2 proteins are polypeptides of 538 amino acids, the sizes of SU and TM subunits are different after processing. It is generally believed that the processed SU and TM subunits form an unactivated complex until the interaction between SU and receptor, which allows TM to resume its fusion activity. Indeed, in the unactivated state of murine leukemia virus (MLV) Env, a typical disulphide-isomerase-active motif, CX_2C, in the SU subunit has been reported to form a disulphide bond with the last cysteine residue in a CX_6CC motif in the TM subunit [24]. Recently, Wallin et al. [25] have further demonstrated that receptor binding induces isomerization of the disulphide bond between SU and TM to release SU and form an intrasubunit disulphide bond in the CX_2C motif. Under this situation, the fusion function of TM is activated. Because syncytin 1 and 2 proteins have both motifs, we speculated that formation and isomerization of a disulphide bond between CX_2C and CX_6CC are essential for their biosynthesis and fusion function. In the present study, mutagenesis of the CX_2C motif in syncytins 1 and 2 not only abolished their fusion activities but also imposed a dominant negative effect on the wild-type syncytins 1 and 2, respectively. Of note, mutation of the first cysteine in the CX_2C motif (C186 in syncytin 1 and C43 in syncytin 2) has a stronger effect in blocking the fusion activity of the wild type, suggesting that this mutant may efficiently compete with the wild-type SU for TM to form a mutant unactivated complex, which will not undergo isomerization to activate the fusion activity of TM. Additionally, our domain swapping experiments indicated that the SU and TM subunits of syncytins 1 and 2 are not interchangeable in terms of fusion activity. Because the CX_2C motif is located in the middle of the SU of syncytin 1 and in the N-terminal portion of the SU of syncytin 2, we also speculate that swapping the SU and TM subunits may affect the formation and isomerization of the disulphide bond essential for biosynthesis and fusion function. This speculation is further substantiated by our observation that Syn1C186A has no effect on blocking syncytin 2 processing, and likewise with Syn2C43A on syncytin 1.

This study also demonstrated that furin is involved in the proteolytic cleavage of syncytins 1 and 2, because furin inhibitor I blocked the cleavage and, therefore, the syncytin 1- and syncytin 2-mediated cell-cell fusion. The consensus furin cleavage site is positioned after the C-terminal R residue in the sequence R/K-R or R-X_2-R [29]. Accordingly, the furin cleavage sites in syncytins 1 and 2 are R-N-K-R and R-V-R-R, respectively. The differences in the sequences of these two sites seem to have no significant effect on the processing of precursor proteins, because modification of one site into the other does not affect the level of TM (data not shown). Furin is a member of the proprotein convertase family of serine proteases, including PC1/3, PC2, and PC4, to name a few [29]. When transiently expressed in the furin-defective LoVo cells, we found that the processing of syncytin 1 and 2 polypeptides was not efficient enough to confer cell-cell fusion (Fig. 4, C and D). However, enhanced processing of both precursor proteins and significant cell-cell fusion were detected when functional furin was coexpressed in LoVo cells (Fig. 4, C and D). Therefore, furin is a major protease in the conversion of syncytin proproteins into mature fusogenic proteins, and cell-cell fusion ensues when a sufficient level of mature fusogenic protein is available. Genetic ablation of FURIN in mice is embryonically lethal between 10.5 and 11.5 days postcoitum due to severe ventral closure defects and the failure of heart development [30]. Interestingly, the allantois of the FURIN-deficient embryo fails to fuse with the chorion [30], suggesting that the functional fusion protein(s) involved in the process may be missing. It will be intriguing to test whether processing of syncytins A and B is abolished and responsible for the defective chorioallantoic fusion in FURIN-deficient embryos. Overall, our study revealed new information about multiple aspects of the biology of syncytin 2, including the regulation of its gene expression, the roles of the CTM domain and motifs in the biosynthesis and protein function of syncytin 2, and the differential properties of syncytin 1 and 2 proteins.

ACKNOWLEDGMENT

We thank Dr. Harry Wilson of Academia Sinica for manuscript editing.

REFERENCES

1. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, et al. Initial sequencing and analysis of the human genome. Nature 2001; 409:860–921.
2. Griffiths DJ. Endogenous retroviruses in the human genome sequence. Genome Biol 2001; 2:REVIEWS1017.–1017.5.
3. de Parseval N, Heidmann T. Human endogenous retroviruses: from infectious elements to human genes. Cytogenet Genome Res 2005; 110: 318–332.
4. Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC Jr, McCoy JM. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 2000; 403:785–789.
5. Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M, Rabreau M, Evain-Brion D, Mallet F. Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. Mol Cell Biol 2003; 23:3566–3574.
6. Dunlap KA, Palmarini M, Varela M, Burghardt RC, Hayashi K, Farmer JL, Spencer TE. Endogenous retroviruses regulate periimplantation placental growth and differentiation. Proc Natl Acad Sci U S A 2006; 103:14390–14395.
7. Lee X, Keith JC Jr, Stumm N, Moutsatsos I, McCoy JM, Crum CP, Genest D, Chin D, Ehrenfels C, Pijnenborg R, van Assche FA, Mi S. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. Placenta 2001; 22:808–812.
8. Knerr I, Beinder E, Rascher W. Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome. Am J Obstet Gynecol 2002; 186: 210–213.
9. Chen CP, Wang KG, Chen CY, Yu C, Chuang HC, Chen H. Altered placental syncytin and its receptor ASCT2 expression in placental development and pre-eclampsia. BJOG 2006; 113:152–158.
10. Antony JM, van Marle G, Opii W, Butterfield DA, Mallet F, Yong VW, Wallace JL, Deacon RM, Warren K, Power C. Human endogenous retrovirus glycoprotein-mediated induction of redox reactants causes oligodendrocyte death and demyelination. Nat Neurosci 2004; 7:1088–1095.
11. Bjerregaard B, Holck S, Christensen IJ, Larsson LI. Syncytin is involved in breast cancer-endothelial cell fusions. Cell Mol Life Sci 2006; 63:1906–1911.

12. Strick R, Ackermann S, Langbein M, Swiatek J, Schubert SW, Hashemolhosseini S, Koscheck T, Fasching PA, Schild RL, Beckmann MW, Strissel PL. Proliferation and cell-cell fusion of endometrial carcinoma are induced by the human endogenous retroviral Syncytin-1 and regulated by TGF-beta. J Mol Med 2007; 85:23–38.
13. Larsson LI, Holck S, Christensen IJ. Prognostic role of syncytin expression in breast cancer. Hum Pathol 2007; 38:726–731.
14. Blaise S, de Parseval N, Benit L, Heidmann T. Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. Proc Natl Acad Sci U S A 2003; 100:13013–13018.
15. Seifarth W, Skladny H, Krieg-Schneider F, Reichert A, Hehlmann R, Leib-Mosch C. Retrovirus-like particles released from the human breast cancer cell line T47-D display type B- and C-related endogenous retroviral sequences. J Virol 1995; 69:6408–6416.
16. Dupressoir A, Marceau G, Vernochet C, Benit L, Kanellopoulos C, Sapin V, Heidmann T. Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae. Proc Natl Acad Sci U S A 2005; 102:725–730.
17. Chang C, Chen PT, Chang GD, Huang CJ, Chen H. Functional characterization of the placental fusogenic membrane protein syncytin. Biol Reprod 2004; 71:1956–1962.
18. Andersen KB. A domain of murine retrovirus surface protein gp70 mediates cell fusion, as shown in a novel SC-1 cell fusion system. J Virol 1994; 68:3175–3182.
19. Report of the National High Blood Pressure Education Program Working Group on high blood pressure in pregnancy. Am J Obstet Gynecol 2000; 183(suppl 1):S1–S22.
20. Yu C, Shen K, Lin M, Chen P, Lin C, Chang GD, Chen H. GCMa regulates the syncytin-mediated trophoblastic fusion. J Biol Chem 2002; 277:50062–50068.
21. Keryer G, Alsat E, Tasken K, Evain-Brion D. Cyclic AMP-dependent protein kinases and human trophoblast cell differentiation in vitro. J Cell Sci 1998; 111:995–1004.
22. Takahashi S, Kasai K, Hatsuzawa K, Kitamura N, Misumi Y, Ikehara Y, Murakami K, Nakayama K. A mutation of furin causes the lack of precursor-processing activity in human colon carcinoma LoVo cells. Biochem Biophys Res Commun 1993; 195:1019–1026.
23. Takahashi S, Nakagawa T, Kasai K, Banno T, Duguay SJ, Van de Ven WJ, Murakami K, Nakayama K. A second mutant allele of furin in the processing-incompetent cell line, LoVo. Evidence for involvement of the homo B domain in autocatalytic activation. J Biol Chem 1995; 270: 26565–26569.
24. Pinter A, Kopelman R, Li Z, Kayman SC, Sanders DA. Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes. J Virol 1997; 71:8073–8077.
25. Wallin M, Ekstrom M, Garoff H. Isomerization of the intersubunit disulphide-bond in Env controls retrovirus fusion. EMBO J 2004; 23:54–65.
26. Kudaka W, Oda T, Jinno Y, Yoshimi N, Aoki Y. Cellular localization of placenta-specific human endogenous retrovirus (HERV) transcripts and their possible implication in pregnancy-induced hypertension. Placenta 2008; 29:282–289.
27. Malassine A, Blaise S, Handschuh K, Lalucque H, Dupressoir A, Evain-Brion D, Heidmann T. Expression of the fusogenic HERV-FRD Env glycoprotein (syncytin 2) in human placenta is restricted to villous cytotrophoblastic cells. Placenta 2007; 28:185–191.
28. Mangeney M, Renard M, Schlecht-Louf G, Bouallaga I, Heidmann O, Letzelter C, Richaud A, Ducos B, Heidmann T. Placental syncytins: genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins. Proc Natl Acad Sci U S A 2007; 104: 20534–20539.
29. Taylor NA, Van De Ven WJ, Creemers JW. Curbing activation: proprotein convertases in homeostasis and pathology. FASEB J 2003; 17:1215–1227.
30. Roebroek AJ, Umans L, Pauli IG, Robertson EJ, van Leuven F, Van de Ven WJ, Constam DB. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. Development 1998; 125: 4863–4876.