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

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ABSTRACT

Syncytin is an envelope protein of the human endogenous retrovirus family W (HERV-W). Syncytin is specifically expressed in the human placenta and mediates trophoblast cell fusion into the multinucleated syncytiotrophoblast layer. It is a polypeptide of 538 amino acids and is predicted to be posttranslationally cleaved into a surface (SU) subunit and a transmembrane (TM) subunit. Functional characterization of syncytin protein can aid understanding of the molecular mechanism underlying syncytin-mediated cell fusion. In this report, we studied the structure-function relationship of syncytin in 293T and HeLa cells transiently expressing wild-type syncytin or syncytin mutants generated by linker scanning and deletion mutagenesis. Of the 22 linker-inserted mutants, mutants InS(51), InV(139), InE(156), InS(493), InA(506), and InL(529) were fusogenic, suggesting that regions around amino acids S^{51}, V^{139}, and E^{156} in the SU subunit and S^{493}, A^{506}, and L^{529} in the cytoplasmic domain (CTM) of syncytin are flexible in conformation. Of the 17 deletion mutants, nine mutants with deletions in the region from amino acids 479 to 538 were fusogenic. The deletion mutant DelI(480), containing only the first four amino acid residues in the cytoplasmic domain, had enhanced fusogenic activity in comparison with the wild-type. In addition, two heptad repeat regions (HRA and B) were defined in the TM subunit of syncytin. A peptide inhibitor derived from the C-terminal heptad repeat region (HRB) was shown to potently inhibit syncytin-mediated cell fusion. Our results suggest that the cytoplasmic domain of syncytin is not essential for syncytin-mediated fusion but may play a regulatory role, and an intramolecular interaction between HRA and B is involved in the fusion process.

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

INTRODUCTION

Cell-cell fusion is required in both normal developmental and pathological processes. For example, a diploid zygote is formed after the fusion of gametes at the beginning of development. Furthermore, maturation of the mammalian embryo requires intercellular fusion to form a syncytiotrophoblast layer, myotubes, and osteoclasts. In pathological processes, macrophages fuse to form multinucleated giant cells during inflammation, and fusion of the viral membrane with the host cell membrane facilitates viral entry into cells. In the human placenta, cytotrophoblasts fuse with each other to form a thin and multinucleated syncytiotrophoblast layer [1]. Fusion of cytotrophoblasts may be an important measure to meet the requirement of physiological conditions during pregnancy. Indeed, the syncytiotrophoblast layer (syncytium) is responsible for transporting nutrients and gases and producing hormones, such as placental lactogen and chorionic gonadotroph [1].

Recently, a fusogenic membrane protein called syncytin was identified and shown to be specifically expressed in the syncytiotrophoblast layer of human placenta [2]. Syncytin is encoded by an *envelope* (*Env*) gene of the human endogenous retrovirus family W (HERV-W). It is a polypeptide of 538 amino acids and is predicted to be posttranslationally cleaved into a surface (SU) subunit and a transmembrane (TM) subunit by analogy to other retroviral Env proteins. Syncytin has been demonstrated to mediate cell fusion in a variety of mammalian cell lines, including BeWo, COS, and 293 cells [2, 3]. As in the fusion process of retroviral Env protein, syncytin binds its cognate receptor to initiate the fusion process. Currently, two human sodium-dependent amino acid transporters, ASCT1 and 2, have been reported as the putative syncytin receptors [4]. Murine cells expressing these transporters were able to form syncytia when the syncytin expression plasmid was also introduced into these cells. However, a direct interaction between syncytin and these transporters has not been demonstrated. Furthermore, it is not known if any auxiliary receptor is involved in the interaction between syncytin and these transporters.

A maturation process is required during the biosynthesis of fusogenic retroviral Env glycoproteins [5]. It is believed that protease(s) in the *trans*-Golgi compartment cleaves the Env protein precursor to release the SU and TM subunits, which then associate together and traffic to the cell surface. The fusion peptide located at the amino terminus of TM interacts with the target cell membrane during the interaction between SU and retroviral receptor. However, currently, neither the biosynthetic pathway of syncytin nor the structure-function relationship of syncytin is clear. Both issues are important for understanding the molecular mechanism underlying syncytin-mediated cell fusion. In this article, we approached these issues by functional characterization of syncytin protein using linker-scanning and deletion mutagenesis. Twenty two linker-inserted and 17 deletion mutants were generated and assessed for their fusogenic activities in 293T and HeLa cells, which do not normally express syncytin. The cytoplasmic domain of syncytin is not essential for syncytin-mediated cell fusion but may play a regulatory role in the process and an intact N-terminal region of syncytin is required for fusion, with the exception of three flexible sites (S^{51}, V^{139}, and E^{156}). In addition, two intervening heptad repeat regions (HRA and HRB) were identified in the TM subunit. The involvement

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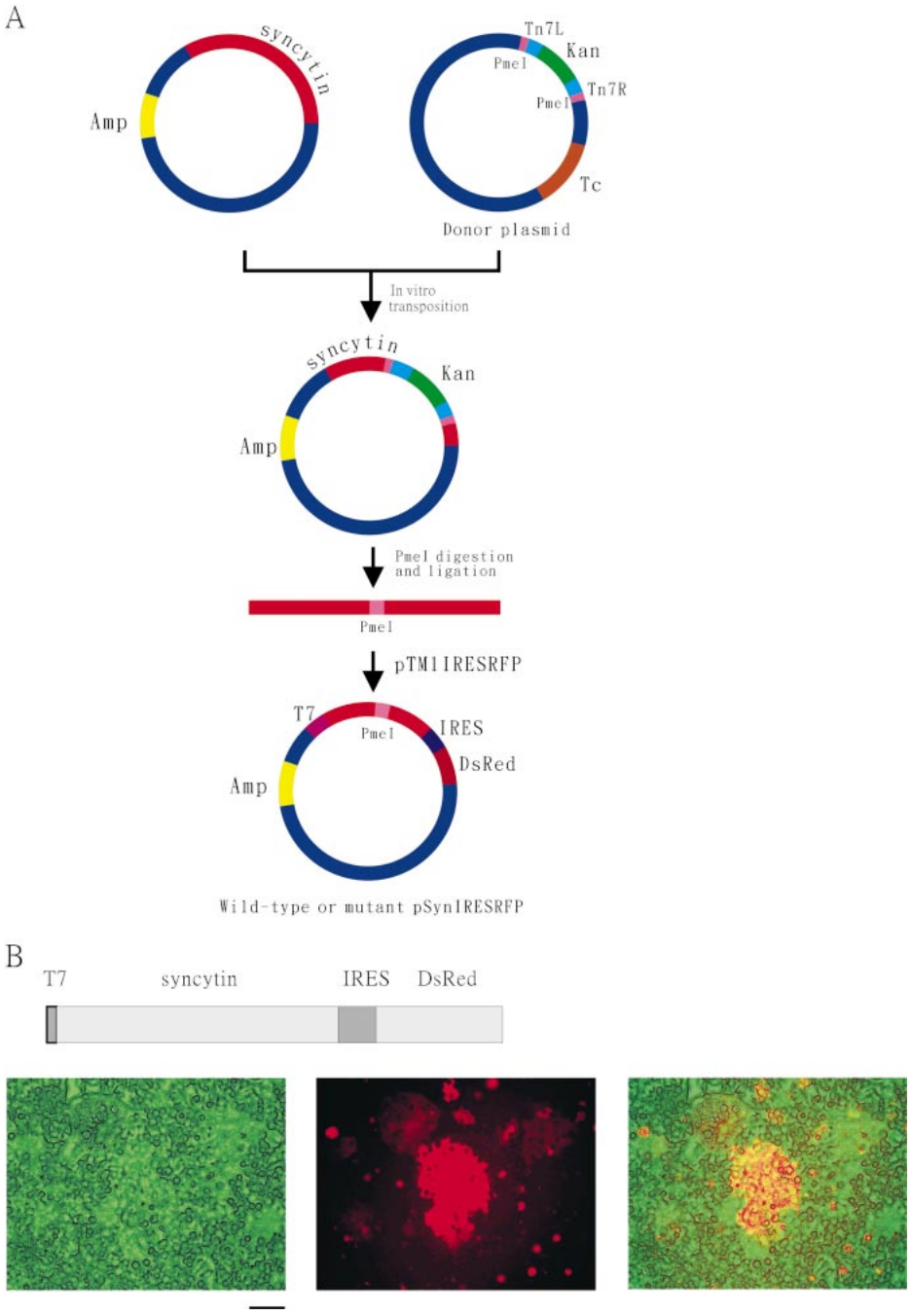

FIG. 1. Syncytin fusion assay. **A**) Schematic representation of wild-type and mutant syncytin expression plasmids. Plasmid construction was performed as described in *Materials and Methods*. T7, T7 RNA polymerase promoter; IRES, internal ribosome entry site; DsRed, *Discosoma sp.* red fluorescent protein. **B**) Cell fusion mediated by syncytin. 293T cells in six-well culture plates were infected with vTF7-3, followed by transfection of 1 μg of pSyn-IRESRFP. Cells showing red fluorescence suggest coexpression of syncytin and RFP (middle panel). These cells underwent cell-cell fusion and formed syncytia, characterized by patches of red fluorescence. A composite bright-field image (left panel) and fluorescence image is also presented (right panel). Bar = 100 μm.

of the heptad repeat regions in syncytin-mediated cell fusion is further discussed in this study.

MATERIALS AND METHODS

Plasmid Construction and Mutagenesis

Syncytin cDNA was subcloned into the pIRESDsRed plasmid (BD Biosciences Clontech, Palo Alto, CA), which harbors a red fluorescent protein (RFP) downstream of an IRES (internal ribosomal entry site) element. The resultant plasmid containing syncytin-IRES-DsRed, was further cleaved and subcloned into pTM1 plasmid (kindly provided by Dr. Bernard Moss, National Institutes of Health) to generate pSynIRESRFP. Linker-scanning mutagenesis was performed using a commercial kit based on the in vitro Tn7-based transposition system (NEB, Beverly, MA) (Fig. 1A). In brief, a plasmid containing the syncytin cDNA fragment was used as the target template for in vitro transposition, to which a donor plasmid containing a kanamycin selection marker flanked by two attachment sites (Tn7L and R) for transposase was added. Transposition was initiated after addition of transposase and the reaction mixture was incubated at 25°C for 30 min. The reaction mixture was then transformed into HB101, and kanamycin-resistant colonies were selected. Plasmids prepared from kanamycin-resistant colonies were digested with *Pme* I to cleave off the selection marker. The resultant linearized syncytin plasmid was self-ligated, resulting in a 15-base pair (bp) insertion in syncytin cDNA. Sequence for the 15-bp insertion is TGTTTAAACA$(N)_5$, where $(N)_5$ are the duplicated nucleic acid residues at the insertion site. After sequencing, mutant syncytin cDNA fragments were further cleaved and substituted for the wild-type syncytin fragment in pSynIRESRFP.

Cell Culture and Transfection

All cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). 293T or HeLa cells were maintained at 37°C in minimal essential medium alpha medium, 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. For syncytin expression, 293T or HeLa cells in six-well culture plates were infected with vTF7–3 [6], a recombinant vaccinia virus expressing T7 RNA poly-

merase, at a multiplicity of infection of 10 viral particles per cell for 90 min. After infection, cells were transfected with 1 μg of expression plasmid using the calcium phosphate coprecipitation method for 293T cells or the Geneporter system (Gene Therapy Systems, San Diego, CA) for HeLa cells.

Fusion Assay of Syncytin Protein

We began to monitor syncytin-mediated cell fusion 12 h after transfection by tracing RFP-expressing cells, which coexpress syncytin. In addition, red fluorescence in transfected cells was monitored to estimate transfection efficiency. As fusion proceeded, patches of red fluorescence were observed under the microscope and scored as fusion events (Fig. 1B). Fusion events were also scored under bright-field microscopy as 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 syncytin 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 [7]. The Student t-test was used to determine statistical significance for differences between means. A P value of less than 0.05 was considered significant.

Peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry [8], purified by HPLC on a C_{18} column, and verified by mass spectrometry. All peptides were acetylated at the N-terminus and amidated at the C-terminus to enhance biological half-life. For inhibition assays, transfected 293T cells in 24-well plates were treated with peptides at the indicated concentration for 24 h. Multinucleated syncytia were counted by microscopic examination at 40× magnification, which allowed visualization of the entire well in a single field.

Metabolic Labeling, Immunoprecipitation, and Cell Surface Biotinylation

Cells were infected with vTF7-3 and transfected with the indicated expression plasmid as described above. Twelve hours after transfection, cells were starved with DMEM deficient in methionine and cysteine (DMEM-met/-cys) for 45 min and then pulse-labeled with 100 μCi of ^{35}S-methionine and -cysteine in DMEM-met/-cys for 45 min. After labeling, cells were washed once with complete DMEM and then chased by incubation in complete DMEM plus 5 μg/ml of methionine and cysteine for the indicated amount of time. For surface biotinylation, chased cells were washed three times with PBS and biotinylated at 4°C for 30 min with 0.5 mg of NHS-SS-biotin (Pierce, Rockford, IL)/ml in PBS. Cells were lysed in RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) and incubated with guinea pig antiserum against the syncytin TM subunit at 4°C overnight. Protein A-agarose beads (Oncogene, Boston, MA) were added to precipitate the antigen-antibody complex. After extensive washing, the precipitated complex was boiled in 10% SDS for 5 min, diluted with RIPA, and incubated with streptavidin-magnetic beads (Polysciences, Warrington, PA) at 4°C overnight. After extensive washing, proteins were analyzed by SDS-PAGE and fluorography.

RESULTS

Syncytin-Mediated Fusion Assay

For functional characterization of syncytin, we established a fusion assay for syncytin using the vaccine virus-T7 expression system [6]. To monitor fusion of syncytin-expressing cells, an expression plasmid named pSyn-IRESRFP was constructed to coexpress the DsRed red fluorescent protein (RFP) and syncytin in 293T cells (Fig. 1, A and B). Twelve hours after transfection, we were able to observe red fluorescence in cultured cells. As shown in Figure 1B, spots of red fluorescence were suggestive of transfected cells expressing syncytin and RFP. Moreover, patches of red fluorescence were observed, indicating fusion between syncytin-expressing cells and neighboring cells. However, different intensities of red fluorescence were noticed in different patches, suggesting that cell fusion did not synchronously proceed throughout the cell population.

Functional Characterization of Syncytin Mutants

To investigate the structure-function relationship of syncytin, we characterized the functional domains of syncytin using a series of syncytin mutants generated by linker-scanning and deletion mutagenesis. As shown in Table 1, 22 linker-scanning and 17 deletion mutants in total were tested in the fusion assay. Of the 22 linker-scanning mutants, only InS(51), InV(139), and InE(156) with linker sequence inserted in the syncytin SU subunit and InS(493), InA(506), and InL(529) with linker sequence inserted in the syncytin TM subunit were fusogenic (Fig. 2A). Therefore, regions around amino acid residues S^{51}, V^{139}, and E^{156} in the amino terminus of syncytin and S^{493}, A^{506}, and L^{529} in the cytoplasmic domain (CTM) of syncytin are flexible in conformation to tolerate insertion of an extra five amino acids. In contrast, mutant InI(337) carrying an extra five amino acids adjacent to the C-terminus of the fusion peptide, without changing the helical structure of fusion peptide, still lost its fusogenicity. In the deletion mutagenesis study, mutants were fusogenic as long as the deletion was limited to the cytoplasmic domain. Of note, the deletion mutant DelI(480), which contains only four amino acid residues of the cytoplasmic domain, was also fusogenic (Fig. 2A). The loss of fusogenic activities in those null mutants was not due to low transfection efficiencies or a low level of transcript because the number of red fluorescent cells and the detection of Northern signals were similar in all mutants tested (data not shown).

Cytoplasmic Domain of Syncytin Regulates its Fusion Activity

While characterizing the deletion mutants, we observed a conspicuous enhancement of fusion in 293T or HeLa cells transfected with the mutant DelI(480) in comparison with the wild-type syncytin. As shown in Figure 3, A and B, fusion occurred to a higher degree in the DelI(480)-transfected cells when compared with the wild-type syncytin-transfected cells. The fusion index of the mutant DelI(480) was almost threefold higher than that of wild-type sycnytin in 293T cells (Fig. 3C). Similar results were observed in HeLa cells (data not shown). To further understand the differential fusogenic activity between wild-type and mutant DelI(480), we analyzed the biosynthesis of both proteins on the cell membranes of transfected HeLa cells. HeLa cells transfected with wild-type or mutant DelI(480) syncytin were pulsed with ^{35}S-methionine and -cysteine and chased for 30, 60, 120, and 240 min. Surface-bound wild-type and mutant syncytin proteins were sequentially biotinylated and precipitated with an antiserum against the TM subunit of syncytin and streptavidin-magnetic beads. As shown in Figure 3D, wild-type and DelI(480) syncytin precursor proteins were detected on cell surfaces 30 min after chasing (lanes 1 and 5). As the length of the chase period increased, more precursor proteins were detected on cell surfaces. Cleaved TM subunits from wild-type and DelI(480) syncytin precursor proteins were detected 120 min after the chase (Fig. 3D, lanes 3 and 7). Considering the number of methionine residues in the wild type and DelI(480) TM subunits, the signals of both TM subunits were not significantly different. Therefore, the differential fusogenic activity between wild-type and DelI(480) syncytin proteins is unlikely due to a differential proteolytic cleavage of the precursor proteins during their biosyntheses. Based on these observations, the cytoplasmic domain of syncytin may have an inhibitory effect on its cell-fusion process.

TABLE 1. Fusion assay of syncytin mutants.

Mutants[a]	ATG[b] no.	Nucleotide sequence[c]	Amino acid sequence[d]	Amino acid no.	Fusion[e]
Insertion mutants					
InD (48)	144	GGA AAT ATT GAT tgt tta aac a**TT GAT** GCC CCA	GNIDclniDAP	D (48)	−
InS (51)	154	GCC CCA TCG Ttg ttt aaa ca**A TCG T**AT AGG	APSlfkqSYR	S (51)	+
InF (62)	186	ACC CCC ACC TTC tgt tta aac a**CC TTC** ACT GCC	TPTFclntFTA	F (62)	−
InI (92)	277	AAA ATG ATT Atg ttt aaa ca**G ATT A**AT CCT	KMImfkqINP	I (92)	−
InC (96)	288	AAT CCT AGT TGT tgt tta aac a**GT TGT** CCT GGA	NPSCclnsCPG	C (96)	−
InM (114)	343	ACT GGT ATG Ttg ttt aaa ca**T ATG T**CT GAT	TGM1fkhMSD	M (114)	−
InQ (123)	369	CAA GAT CAG tgt tta aac a**AT CAG** GCA AGA	QDQclnnQAR	Q (123)	−
InV (139)	418	ACC CGG GTA Ctg ttt aaa ca**G GTA C**AT GGC	TRV1fkqVHG	V (139)	+
InE (156)	469	CTA CAT GAA Atg ttt aaa ca**T GAA A**CC CTC	LHEmfkhETL	E (156)	+
InH (176)	528	GGG CTC CAT tgt tta aac a**TC CAT** GAG GTC	GLHclniHEV	H (176)	−
InL (227)	682	TCC AAT CTG Gtg ttt aaa ca**T CTG G**AA ATA	SNLvfkhLEI	L (227)	−
InC (237)	711	CTC ACC TGT tgt tta aac a**CC TGT** GTA AAA	LTCclntCVK	C (237)	−
InS (275)	826	GGT ACC TCA Gtg ttt aaa ca**C TCA G**CC TAT	GTSvfkhSAY	S (275)	−
InI (337)	1012	ACT GGC ATT Gtg ttt aaa ca**C ATT G**GC GGT	TGIvfkhIGG	I (337)	−
InM (358) Alt	1074	GGG GAC ATG tgt tta aac a**AC ATG** GAA CGG	GDMclnnMER	M (358)	−
InM (358)	1075	GGG GAC ATG Gtg ttt aaa ca**C ATG G**AA CGG	GDMvfkhMER	M (358)	−
InL (400)	1200	TTA TTT TTA tgt tta aac a**TT TTA** GGG GAA	LFLclniLGE	L (400)	−
InK (419)	1258	AAA GTT AAA Gtg ttt aaa ca**T AAA G**AA ATT	KVKvfkhKEI	K (419)	−
InQ (426)	1279	CGA ATA CAA Ctg ttt aaa ca**A CAA C**GT AGA	RIQlfkqQRR	Q (426)	−
InS (493)	1479	ATG CAG TCC tgt tta aac a**AG TCC** AAG ACT	MQSclnkSKT	S (493)	+
InA (506)	1518	CGG CCT GCT tgt tta aac a**CT GCT** AGC CCA	RPAclntAPR	A (506)	+
InL (529)	1588	CAA CCT CTA Ctg ttt aaa ca**T CTA C**TA CGC	QPL1fkhLLR	L (529)	+
Deletion mutants					
DelI (47)	140	CCC GGA AAT ATt gtt taa aca	PGNIcv [stop]	I (47)	−
DelV (102)	305	GGA CTT GGA GTt gtt taa aca	GLGVv [stop]	V (102)	−
DelI (252)	755	TCC CAA TGC ATt gtt taa aca	SQCIv [stop]	I (252)	−
DelA (276)	827	GGT ACC TCA GCt gtt taa aca	GTSAv [stop]	A (276)	−
DelI (320)	959	AGA GTA CCC Att gtt taa aca	RVPIv [stop]	I (320)	−
DelR (384)	1157	AAT CGA AGA GTA tgt tta aac aGT TTT AGA CTT GCT AAC CGC TGA	NRRvclnsfrlanr [stop]	R (384)	−
DelY (407)	1226	TAT TAT GTA tgt tta aac aGT TAA	YYvclns [stop]	Y (407)	−
DelE (416)	1252	ACT GAG TAt gtt taa aca	TEyv [stop]	E (416)	−
DelK (419)	1259	AAA GTT AAA GAt gtt taa aca	KVKdv [stop]	K (419)	−
DelR (427)	1280	CGA ATA CAA CGt gtt taa aca	RIQRv [stop]	R (427)	−
DelA (456)	1367	GGA CCT CTA GCt gtt taa aca	GPLAv [stop]	A (456)	−
DelI (480)	1444	GTC TCT TCC AGA ATC TAG	VSSRI [stop]	I (480)	+
DelT (495)	1489	ATG CAG TCC AAG ACT TAG	MQSKT [stop]	T (495)	+
DelS (507)	1520	CGG CCT GCT AGt gtt taa aca	RPASv [stop]	S (507)	+
DelS (510)	1534	GCT AGC CCA CGA TCT TAG	ASPRS [stop]	S (510)	+
DelP (520)	1562	ACC CCT CCT GAt gtt taa aca	TPPdv [stop]	P (520)	+
DelI (523)	1573	CCT CCT GAG GAA ATC TAG	PPEEI [stop]	I (523)	+

[a] The nomenclature of mutants was based on the type of mutagenesis. In and Del are abbreviations for linker insertion and deletion mutagenesis, respectively. The capital and number in parenthesis after In or Del refer to the mutated amino acid residue and its number in the syncytin polypeptide, respectively.
[b] The number stands for the nucleic acid position where the mutagenesis occurred relative to the translational initiation site.
[c] Nucleic acids in uppercase indicate the sequence flanking the mutation site. Duplicated nucleic acid residues are in bold type. Nucleic acids in lowercase indicate the linker sequence.
[d] Amino acids in uppercase indicate the sequence flanking the mutation site. Amino acids in lowercase indicate the linker sequence. Insertion of the linker sequence resulting in a nonsense mutation is indicated by [stop].
[e] Cell fusion was observed from 12 to 24 h after transfection of expression plasmids. +, Fusion occurred; −, no fusion occurred.

Peptide Inhibition of Syncytin-Mediated Fusion

One of the structural features of retroviral envelope proteins lies in the ectodomain of the TM subunit, whose primary sequence is in part predicted to be extended amphipathic α-helices like the leucine zipper motif [5, 9, 10]. Synthetic peptides derived from these regions have been used to study the relationship between the secondary structure and the fusogenic activity of envelope proteins [11, 12]. Therefore, we analyzed the primary sequence of syncytin using the Lupas algorithm [13, 14] for predicting secondary structure of leucine zipper-like motif. As shown in Figure 2B, two heptad repeat regions (HRA and HRB) were identified in the TM subunit of syncytin. To investigate the functional relationship between these two heptad repeat regions and the fusogenicity of syncytin, three peptides, HRA1, HRA2, and HRB1, located in the HRA and B regions were synthesized (Fig. 4A). As negative controls, two unrelated peptides, H32 and DP178, were also synthesized (Fig. 4A). The former contained three copies of the hemagglutinin tag, whereas the latter was derived from the TM subunit (gp41) of HIV-1 and is a potent inhibitor of HIV-1-mediated cell-cell fusion [12]. We tested if these peptides could block syncytin-mediated cell-cell fusion in 293T cells transiently expressing syncytin. As shown in Figure 4B, H32, DP178, and HRA1 did not block cell fusion; however, HRB1 was a very potent inhibitor that completely blocked cell fusion at 1.6 μg/ml. Compared with HRB1, HRA2 was a less potent inhibitor that, even at a relatively high concentration of 5.2 μg/ml, only inhibited about 35% cell fusion relative to the mock control (Fig. 4B). Together with

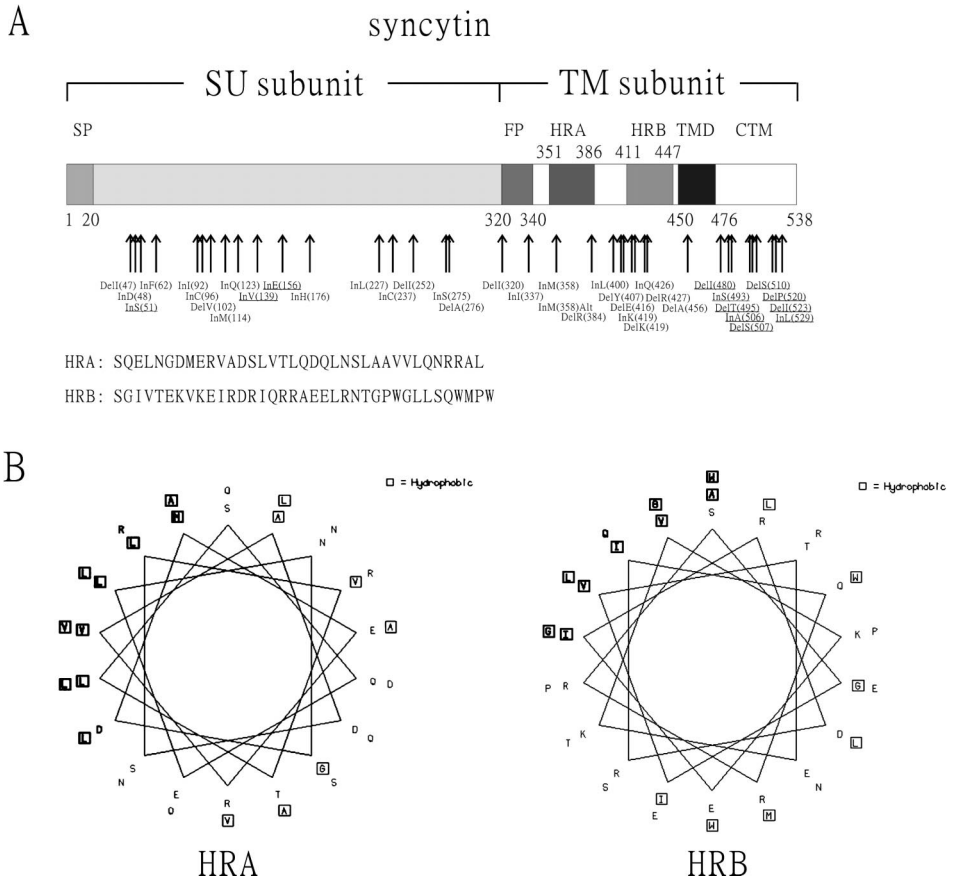

FIG. 2. Schematic summary of fusion assays. **A**) Predicted functional domains of syncytin. The numbers underneath the domain structure indicate the numbering of amino acid in the syncytin polypeptide. SP, Signal peptide; FP, fusion peptide; HR, heptad repeat region; TMD, transmembrane domain; CTM, cytoplasmic domain. Arrows indicate the positions of mutagenesis in the syncytin polypeptide. Nomenclature of mutants is the same as described in Table 1. Mutants of functional fusogenic activity are underlined. Amino acid sequences of HRA and B are listed. **B**) Helical wheel representations of the HR sequences within the ectodomain of syncytin TM subunit. Potential hydrophobic surfaces of HRA and B are highlighted. Sequences were analyzed and diagrams were drawn by the HelicalWheel program of the Genetics Computer Group programs (Madison, WI).

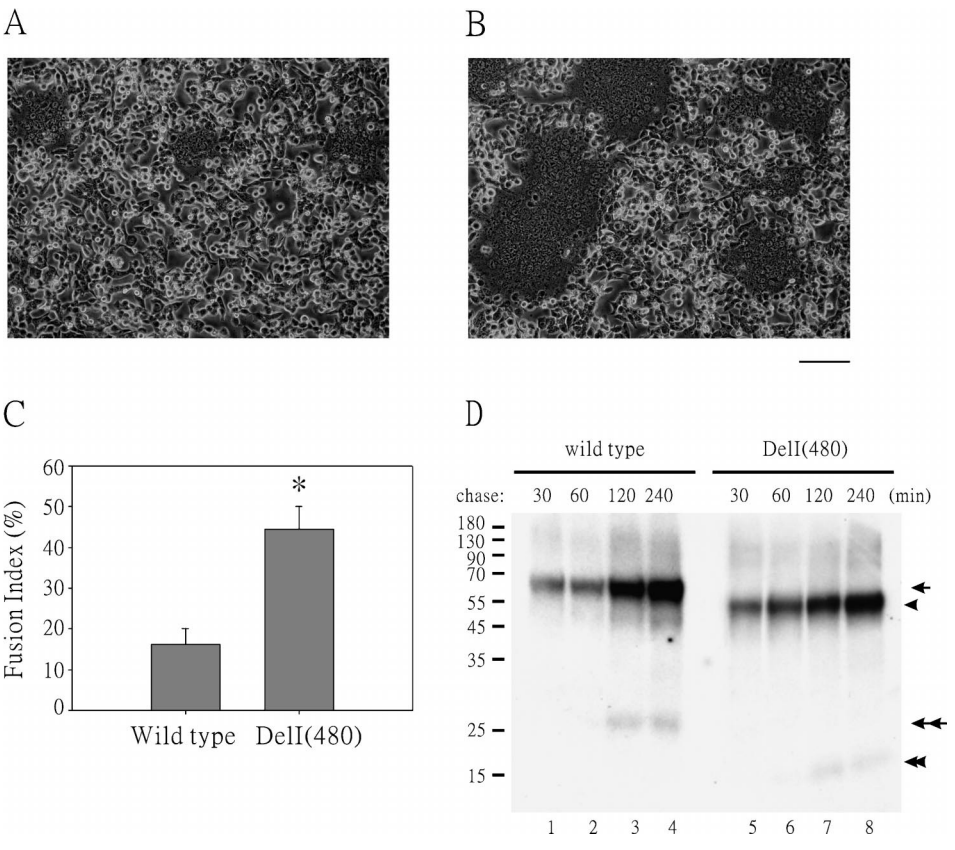

FIG. 3. The CTM of syncytin affects its fusogenic activity. 293T cells transiently expressing wild-type (**A**) or DelI(480) mutant (**B**) syncytin underwent fusion differentially. Bar = 100 μm. DelI(480) mutant syncytin containing only the first four amino acid residues of the CTM has a higher fusion efficiency than wild-type, as indicated by fusion index (**C**). Values are mean and SEM obtained from three independent experiments. Asterisk denotes significant difference (*, $P < 0.05$) between wild-type and DelI(480). **D**) Biosynthesis of wild-type and DelI(480) mutant syncytin proteins on cell surface. HeLa cells transiently expressing wild-type or DelI(480) mutant syncytin were analyzed by pulse-chase experiments for the wild-type and mutant cell surface syncytin proteins as described in *Materials and Methods.* The numbers on the left stand for the molecular mass of protein markers. Arrow and double arrow indicate the precursor syncytin and the TM subunit of wild-type syncytin, respectively. Arrowhead and double arrowhead indicate the precursor syncytin and the TM subunit of the DelI(480) mutant syncytin, respectively. The results shown are from one of three independent experiments.

the findings from the linker-scanning mutagenesis study that mutants InM(358), InM(358)Alt, InK(419), and InQ(426), harboring linker sequences in HRA and B in frame with the polypeptide backbone were not fusogenic (Table 1 and Fig. 2), these results suggested that HRB1 is a specific peptide inhibitor for syncytin-mediated cell-cell fusion and the heptad repeat regions are involved in the fusion process.

DISCUSSION

Syncytin is a placenta-specific retroviral envelope protein involved in trophoblastic fusion [2]. The fusogenic activity of syncytin has been demonstrated in several nonplacental cell lines by transient expression [2, 3]. Moreover, suppression of syncytin expression by antisense oligonucleotides results in a decrease in fusion and differentiation of primary-culture trophoblast cells [15]. Clinically, a lower than normal level of syncytin mRNA is associated with placental dysfunction, including preeclampsia and HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome [16]. Therefore, syncytin is thought to play an important role in the formation of the syncytiotrophoblast layer in human placenta. In this study, we first established an easy and fast fusion assay for syncytin by coexpression of syncytin and red fluorescence protein via a single transcript in a vaccinia virus expression system. We then identified conformationally flexible sites S^{51}, V^{139}, and E^{156} in the N-terminal region of syncytin and S^{493}, A^{506}, and L^{529} in the CTM of syncytin by linker-scanning mutagenesis. Deletion mutagenesis of syncytin revealed that the CTM of syncytin is inhibitory to syncytin-mediated cell fusion. Finally, we discovered that predicted heptad repeat regions in the TM subunit of syncytin are essential for syncytin-mediated cell fusion and that the ectodomain of syncytin TM subunit is vulnerable to sequence change even though the linker sequence is in frame with the polypeptide backbone. Especially, mutants with mutations within the FP (InI[337]) and HR (InM[358]Alt, InK[419], and InQ[426]) regions were found to be nonfusogenic even though the insertion sequence was predicted to be helical in terms of secondary structure.

The inhibitory effect of syncytin CTM on cell fusion is reminiscent of the inhibitory R-peptide in the CTM of the envelope protein of murine leukemia virus (MLV). Deletion of this R-peptide in the MLV envelope protein causes extensive fusion with the formation of large syncytia, indicating that R-peptide inhibits membrane fusion [17, 18]. Indeed, the MLV R-peptide is a fragment of 16 amino acids, which is cleaved by the viral protease during virion maturation [19]. Apart from MLV, R-peptide cleavage has been demonstrated in other viruses such as Mason-Pfizer monkey virus and spleen necrosis virus, which are type D and C retroviruses, respectively [20, 21]. Although it is not known whether a similar R-peptide cleavage is required for the maturation of syncytin, the fusogenicity of DelI(480) was enhanced in the absence of most of CTM, suggesting this region may function in a similar manner to R-peptides. Recently, Lavillette et al. [4] demonstrated that an HERV-W Env mutant (termed HERV-Wcyt16), containing a truncated CTM of 16 amino acids, enhances the infectivity of HIV/HERV-Wcyt16 Env pseudotyped viruses. In their study, this enhancement effect was attributed to the interference of the long CTM in the maturation process of the Env protein precursor. Nevertheless, we did not observe a significant difference between wild-type and DelI(480) syncytin in the maturation process in HeLa (Fig. 3D) and 293T

A

H32: MAYPYDVPDYAGYPYDVPDYAGSYPYDVPDYA
DP178: YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF
HRA1: KLSQELNGDMERVADSLVTLQDQLNSLAAV (349-378)
HRA2: DSLVTLQDQLNSLAAVVLQNRRALDLLTAE (363-392)
HRB1: SGIVTEKVKEIRDRIQRRAEELRNTGPWGL (411-440)

B

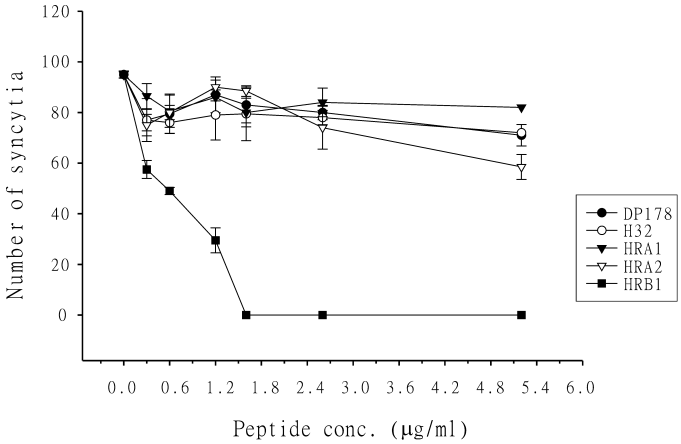

FIG. 4. Blockade of syncytin-mediated cell-cell fusion by peptide inhibitors. **A**) Amino acid sequences of the indicated peptides. Numbers in parentheses indicate the peptide positions in the syncytin polypeptide. **B**) Dose-response of peptide inhibitors against syncytin-mediated cell-cell fusion. 293T cells in 24-well culture plates were treated with the indicated peptides at different concentrations, as denoted in the x-abscissa. Values are mean and SEM obtained from three independent experiments.

(data not shown) cells. This discrepancy may be due to fact that different assay systems (pseudotyped viruses vs. cultured cells) were tested in these two studies. Here we would like to point out that syncytin is a placental membrane protein; therefore, the cultured cell system used in this study is more likely to mimic the physiological condition during the maturation process of syncytin.

Heptad repeat sequences are conserved in the fusion proteins of retroviruses, paramyxoviruses, influenza viruses, and coronaviruses [9]. All of these heptad repeat sequences, when displayed on an α-helical wheel, demonstrate an extensive region containing seven amino acid residue repeats of amino acids in a sequence of *a, b, c, d, e, f, g,* with nonpolar residues in all *a* positions and in most *d* positions. These sequences are predicted to form long amphipathic α-helices. For HIV-1 Env protein, two heptad repeat sequences (HR1 and 2) have been identified in the ectodomain of its TM subunit, gp41 [9–12]. Peptides DP107 and DP178, respectively, derived from HR1 and 2 of HIV-1 Env protein, are potent inhibitors of HIV-1-Env-mediated cell fusion [11, 12]. Furthermore, the stable α-helical structure of the DP107 peptide can be disrupted in the presence of DP178 based on CD studies, suggesting an interaction between the two peptides [12]. The predicted HRA and B regions in the ectodomain of the syncytin TM subunit are highly likely to interact with each other during the cell-cell fusion process because the HRB1 peptide may specifically interact with HRA to block cell fusion. Moreover, insertion of linkers into the HRA region (InM[358] and InM[358]Alt) and the HRB region (InK[419] and InQ[426]) also completely abolished the fusion event, possibly by impeding the interaction between the two regions. Several possibilities may explain the observations in this study that

peptide HRA1 did not block fusion and peptide HRA2 had a low efficiency in blocking fusion. First, both peptides may not interact well with HRB. Second, there may be an intrinsic property causing peptides derived from the HRA region of syncytin to be inefficient inhibitors. As in the HIV-1-Env-mediated cell fusion, the HR1-derived DP107 peptide is also a much less efficient inhibitor compared with the HR2-derived DP178 peptide [12]. Third, HRB may be conformationally inaccessible to HRA peptides.

Finally, this study suggests that amino acids S^{51}, V^{139}, and E^{156} in the SU subunit of syncytin are not critically involved in receptor recognition, binding, and the subsequent conformational rearrangement important for formation of the active TM subunit. Nevertheless, identification of these three amino acid residues may provide a basis for engineering a syncytin polypeptide for therapeutic purposes. Recently, it has been shown that, when expressed in tumor cells, fusogenic envelope protein was able to cause syncytia formation and subsequent cell death [22, 23]. Therefore, targeted expression of the syncytin gene in tumor cells is a feasible gene therapy for tumor growth. To this end, it would be possible to insert into the syncytin polypeptide backbone an epitope or motif, which could specifically interact with surface protein in tumor cells. For example, many tumor cells express a higher level of EGF receptor (EGFR) [24]. Insertion of an EGF motif into syncytin may direct a specific interaction between tumor cells expressing a chimeric protein and EGFR. In this study, three potential motif insertion sites (S^{51}, V^{139}, and E^{156}) were identified in the syncytin SU subunit since insertion of linker sequence in these sites did not affect the fusogenic activity of syncytin. Moreover, because DelI(480) has a higher fusogenicity, engineering or insertion of therapeutic motifs could use the backbone of a DelI(480) polypeptide.

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REFERENCES

1. Benirschke K, Kaufmann P. Pathology of the human placenta. New York: Springer-Verlag; 2001:49–56.
2. 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.
3. Blond JL, Lavillette D, Cheynet V, Bouton O, Oriol G, Chapel-Fernandes S, Mandrand B, Mallet F, Cosset FL. An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. J Virol 2000; 74:3321–3329.
4. Lavillette D, Marin M, Ruggieri A, Mallet F, Cosset FL, Kabat D. The envelope glycoprotein of human endogenous retrovirus type W uses a divergent family of amino acid transporters/cell surface receptors. J Virol 2002; 76:6442–6452.
5. Colman PM, Lawrence MC. The structural biology of type I viral membrane fusion. Nat Rev Mol Cell Biol 2003; 4:309–319.
6. Wyatt LS, Moss B, Rozenblatt S. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology 1995; 210:202–205.
7. 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.
8. Chang CD, Felix AM, Jimenez MH, Meienhofer J. Solid-phase peptide synthesis of somatostatin using mild base cleavage of N alpha-9-fluorenylmethyloxycarbonylamino acids. Int J Pept Protein Res 1980; 15:485–494.
9. Chambers P, Pringle CR, Easton AJ. Heptad repeat sequences are located adjacent to hydrophobic regions in several types of virus fusion glycoproteins. J Gen Virol 1990; 71:3075–3080.
10. Delwart EL, Mosialos G, Gilmore T. Retroviral envelope glycoproteins contain a ''leucine zipper''-like repeat. AIDS Res Hum Retroviruses 1990; 6:703–706.
11. Wild C, Oas T, McDanal C, Bolognesi D, Matthews T. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci U S A 1992; 89:10537–10541.
12. Wild CT, Shugars DC, Greenwell TK, McDanal CB, Matthews TJ. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci U S A 1994; 91:9770–9774.
13. Combet C, Blanchet C, Geourjon C, Deleage G. NPS@: network protein sequence analysis. Trends Biochem Sci 2000; 25:147–150.
14. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. Science 1991; 252:1162–1164.
15. 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.
16. 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.
17. Yang C, Compans RW. Analysis of the cell fusion activities of chimeric simian immunodeficiency virus-murine leukemia virus envelope proteins: inhibitory effects of the R peptide. J Virol 1996; 70:248–254.
18. Yang C, Compans RW. Analysis of the murine leukemia virus R peptide: delineation of the molecular determinants which are important for its fusion inhibition activity. J Virol 1997; 71:8490–8496.
19. Green N, Shinnick TM, Witte O, Ponticelli A, Sutcliffe JG, Lerner RA. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. Proc Natl Acad Sci U S A 2004; 78:6023–6027.
20. Brody BA, Rhee SS, Sommerfelt MA, Hunter E. A viral protease-mediated cleavage of the transmembrane glycoprotein of Mason-Pfizer monkey virus can be suppressed by mutations within the matrix protein. Proc Natl Acad Sci U S A 1992; 89:3443–3447.
21. Bobkova M, Stitz J, Engelstadter M, Cichutek K, Buchholz CJ. Identification of R-peptides in envelope proteins of C-type retroviruses. J Gen Virol 2002; 83:2241–2246.
22. Bateman AR, Harrington KJ, Kottke T, Ahmed A, Melcher AA, Gough MJ, Linardakis E, Riddle D, Dietz A, Lohse CM, Strome S, Peterson T, Simari R, Vile RG. Viral fusogenic membrane glycoproteins kill solid tumor cells by nonapoptotic mechanisms that promote cross presentation of tumor antigens by dendritic cells. Cancer Res 2002; 62:6566–6578.
23. Higuchi H, Bronk SF, Bateman A, Harrington K, Vile RG, Gores GJ. Viral fusogenic membrane glycoprotein expression causes syncytia formation with bioenergetic cell death: implications for gene therapy. Cancer Res 2000; 60:6396–6402.
24. Gullick WJ. Type I growth factor receptors: current status and future work. Biochem Soc Symp 1998; 63:193–198.