Trophoblast fusion in placenta is an important event for preservation of a healthy pregnancy. This process takes place throughout the pregnancy and is crucial for the formation of syncytiotrophoblast layer. Syncytin-1 and syncytin-2 are strong candidate regulators of fusion from retroviral origin. Syncytin-A and syncytin-B are other candidates from retroviral origin in Muridae. The active role of syncytin in driving fusion of trophoblast has been identified, but its fusion mechanism is still unclear. As an intact retroviral envelope protein, syncytin-A shares similar structure profiling with other viral envelope fusion proteins, especially in the regions of N- and C-terminal heptad repeats (NHR and CHR, respectively). In this paper, we showed that SynA 1 + 2 of syncytin-A (residues 445–536, including predicted NHR, CHR, and a natural linker) could form trimer and exhibited significant α-helix structure and high thermo-stability. Limited proteolysis result identified a stable protease-resistant core of SynA 1 + 2, which was in good agreement with computational modeling data. NHR and CHR could interact with each other in vitro, too. Different from the previous studies, the disulfide-bonded linker was apparently vital to the stability of fusion core structure. By biological assays, NHR was shown to be inhibitive to cell-cell fusion, with IC_{50} value about 5.4 μM, but CHR seemed to have no inhibitory activity even at 50 μM. From both biochemical and functional data, we first gave an explanation how syncytin-A mediated cell fusion. The insight into the mechanism of syncytin-A-mediated cell-cell fusion may provide a crucial clue to placental cytotrophoblast morphogenesis.

Endogenous retroviruses (ERVs) most probably originated by germ-line infection of the exogenous counterparts in remote
have suggested that specific inhibition of syncytin expression in the primary cytotrophoblasts using antisense oligonucleotides reduces syncytia formation (11, 12). Thus, the ERVs may play an important role in placental morphogenesis: converting the cytotrophoblast into syncytiotrophoblast (19, 20).

Like other class I integral membrane fusion proteins, including the envelope proteins of influenza virus (hemagglutinin HA$_{3}$) (23, 24), MoMLV (TM) (25), HTLV (gp21) (26), Ebola virus (GP2) (27, 28), HIV-1 (gp41) (29–31), and SIV (gp41) (32, 33), syncytin-A protein exhibits characteristic signatures: a proteolytic cleavage site between the surface subunit and transmembrane (TM) subunit, a fusion peptide located just next to the cleavage site, and a hydrophobic domain as a putative fusion core (including NHR, CHR and a natural linker) following the fusion peptide (Fig. 1). Crystal and solution nuclear magnetic resonance structures of TM domains in some viruses, such as HIV-1, Ebola, and so on, show that NHRs form an inner trimer and CHRs pack in an antiparallel orientation in the grooves between NHR monomers (24, 28, 31, 32, 34, 35). Formation of six-helix bundle is believed to pull virus and target membranes together to create a fusion pore. Peptides from the C-helix of HIV-1 potentially inhibit membrane fusion by binding in the grooves of viral N-helix trimers and disrupting the formation of the six-helix bundle. N-helix peptides also inhibit HIV-1 fusion, albeit at 10–100-fold higher concentrations than those of C-helix peptides (36–39).

Taken together, we suggest that syncytin-A may share a common mechanism mediating fusion like other membrane fusion proteins. By homology modeling, we found syncytin-A fusion core protein was much more like the envelope proteins of MoMLV and HTLV with an extended coiled-coil folding back and interacting with a portion of NHR helix trimer. In this study, we identified and characterized the fusion core of syncytin-A and suggested a potential fusion mechanism in the placental formation.

**EXPERIMENTAL PROCEDURES**

**Amino Acids Sequence Analyses of Syncytin-A and Plasmid Constructions**—Syncytin-A protein was analyzed for the probability to form α-helix coiled-coil structures by use of the COILS program. For SynA 1 + 2 (residues 445–536), we amplified the region from amino acids 445 to 536 in syncytin-A from one individual genomic DNA (PCR primers: 5′-GCA GCC TCT GTA ATC CAA CAG CGT CTG-3′ and 5′-GAT TGC GAG TCA AAT CCA AGA AGA AGA AGA AGT ACC CAG TTC AGA AGC ACG TTC ACG TAC CGT TTT-3′). NHR and CHR were cloned into the BamHI/EcoRI site of the pGEX-6p-1 bacterial expression vector, too.

**Protein Expression and Purification—Escherichia coli** strain BL21(DE3) transformed by recombinant pGEX-6p-1 plasmids was grown at 37 °C in 2×YT medium (1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) NaCl) to an optical density of 0.8–1.0 (A$_{590}$). Then it was induced with 1 mM isopropyl β-d-thiogalactopyranoside at 22 °C (for SynA 1 + 2 peptide) and 37 °C (for NHR peptide and CHR peptide) for 8 h. Bacterial cells were harvested and re-suspended in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.5). Triton X-100 was then added to a final concentration of 1% and the bacterial cells were lysed by sonication at 0 °C. Lysate was subsequently clarified by centrifugation at 12,000 × g for 30 min at 4 °C. The clarified supernatants were passed through glutathione-Sepharose 4B column (equilibrated with PBS). The GST fusion protein-bound column was washed by PBS over 10 column volumes and eluted with glutathione elution buffer (10 mM reduced glutathione, 50 mM Tris-Cl, pH 8.0) for 1 column volume. The GST fusion proteins were then cleaved by GST fusion rhinovirus 3C protease (provided by Drs. K. Hudson and J. Heath) at 5 °C for 16 h in the cleavage buffer (50 mM Tris-Cl, pH 7.0, 150 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 8.0). Peptides were further purified by reverse-phase high performance liquid chromatography (HPLC) (Waters 1525) using a C18 column (7.8 × 300 nm column) with a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. The purified peptides were freeze-dried overnight and then stored at −20 °C for further use.

**Size Exclusion HPLC Analysis**—The purified SynA 1 + 2 was loaded into the BioSuite™ 125-5 μm HR SEC column on HPLC (Waters 1525) chromatography system to assess oligomer formation. PBS, pH 7.5, was used as the mobile phase with a flow rate of 1 ml/min. The ultraviolet absorbance at 215 nm was recorded. A gel-filtration of standard consisting of γ-globulin (158 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B$_{12}$ (1.35 kDa) (Sigma) was used to define the molecular weight of SynA 1 + 2.

**Chemical Cross-linking**—SynA 1 + 2 in 50 mM Hepes, pH 8.3, 100 mM NaCl was crosslinked with ethylene glycol-bis-succinimidyl succinate (EGS) (Sigma). The reaction system was incubated at room temperature for 2 h and then quenched with 50 mM glycine. Cross-linked product was analyzed on SDS-PAGE.

**Proteolysis Protection Experiments**—The proteolysis reactions were performed with SynA 1 + 2 and proteinase K (1/50 (w/w) proteinase K/SynA 1 + 2) for 2 h at 4 °C. Samples were immediately subjected to Tricine SDS-PAGE analysis. Protease-resistant fragments were separated from the gel and characterized by mass spectrometry (Voyager DE STR MALDITOF, Applied Biosystems).

**Circular Dichroism Spectroscopy**—NHR was incubated with CHR at 37 °C at equimolar concentration in phosphate-buffered saline, pH 7.4, for 30 min. The isolated SynA 1 + 2, NHR, and CHR were also tested in the same buffer. CD spectra were acquired on a J-810 spectropolarimeter (Jasco, Easton, MD) at
room temperature with a bandwidth of 2 nm, path length of 0.1 cm, response time of 1.0 s, and scanning speed of 50 nm/min. The spectra were corrected by the substraction of a blank corresponding to the solvent. For the function of temperature, wavelength spectra were determined by monitoring the change in CD spectroscopy signal at 222 nm in the range of 25–95 °C. To follow the unfolding in guanidine hydrochloride, we incubated peptides in the denaturant buffer at concentrations of 0.5–6.0 M for 30 min at room temperature, and [θ]222 was plotted versus the guanidine hydrochloride concentrations. In acidic denature experiment, peptides were incubated in PBS buffer with different pH values for 30 min at room temperature, and [θ]222 was plotted versus the different pH values.

Native-PAGE—The isolated NHR, the isolated CHR, and NHR/CHR mixtures at the indicated concentrations were mixed with glycine native sample loading buffer and were then loaded on a 15% glycine gel with a glycine running buffer, pH 8.9. Gel electrophoresis was carried out with constant 150 V at 4 °C for 1.5 h. The gel was then stained with Coomassie Blue.

Molecular Modeling—The molecular model of SynA 1 + 2 protein (residues 447–528) was based on the sequence similarities with other membrane fusion proteins. The trimer structure of NHR was modeled by SwissPDB-Viewer and SWISS-MODEL server, using the structure of HTLV-1 gp21 ectodomain (Protein Data Bank ID: 1MG1) as the template. The additional linker and CHR structure were built by MODELLER 7V7, based on the corresponding regions in the structures of MoMLV (Protein Data Bank ID: 1MOF) and Ebola TM (Protein Data Bank ID: 2EBO), respectively. The minimization and further molecular dynamics simulation procedures were performed by the sander module of AMBER 8 package to remove any bad contact introduced during the modeling process.

Cell Culture and Fusion Assay of Syncytin-A Protein—293T cells were used as target cells. For transfection, cells (~1 × 10^5) were seeded onto the 24-well plate the day before transfection. The monolayers were transfected with 200 ng of the pHCMV-G plasmid expressing syncytin-A envelope protein (gift from Thierry Heidmann, Paris) and 100 ng of pEGFP-C1 plasmid (BD Biosciences) by using calcium phosphate precipitation. GFP fluorescence in transfected cells was monitored to estimate transfection and fusion efficiency. Plates were inspected for cell-cell fusion effect 24–48 h after transfection. Syncytia were visualized by GFP fluorescence and also counted by nucleic staining (4',6-diamidino-2-phenylindole staining method). The fusion index was calculated as [(N – S)/T] × 100, 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. For inhibition assays, transfected 293T cells in 24-well plates were treated with peptides at indicated concentration 5 h after transfection and then assay peptide activity after 24–48 h.

RESULTS
To study syncytin-A protein, we did sequence alignment analyses first and the results were generated by BioEdit version 7.0.1, suggesting that syncytin-A exhibited most features of membrane fusion proteins, as illustrated in Fig. 1. The characteristic sequences are as follows: 1) a consensus cleavage site,
RNKR, which separates the two characteristic glycoprotein domains, surface subunit and TM subunit, 2) a hydrophobic domain as a fusion core following the fusion peptide, including N-terminal heptad repeat and C-terminal heptad repeat, and 3) a conserved Cx6CC motif in the putative linker region. The high degree conservation of transmembrane subunit regions of syncytin-A protein and other class I fusion proteins suggests that these domains may play a similar role in the fusion mechanism.

According to previous studies, many viral envelope fusion proteins such as hemagglutinin HA2 of influenza virus (23, 24), TM of MoMLV (25), gp21 of HTLV (26), GP2 of Ebola virus (27, 28), gp41 of HIV-1 (29–31), and gp41 of SIV (32, 33) have a similar structure, the trimer of hairpins. To investigate whether syncytin-A contains fusion core similar to HIV-1 gp41 and so on, we predicted the putative coiled-coil region of syncytin-A protein by use of the COILS program. To study whether the fusion core region predicted by the above program could form helical trimer of hairpins, we expressed a single chain protein, SynA 1 + 2, corresponding to residues 445–536 of syncytin-A protein and including the predicted NHR and CHR and a natural linker with a conserved Cx6CC motif (Fig. 1B). SynA 1 + 2 formed a trimer in PBS buffer, pH 7.4, as judged by size exclusion HPLC (Fig. 2A). The elution time, relative to molecular mass standards, gave an apparent molecular mass of 32.7 kDa, in good agreement with the theoretical molecular mass of 31.8 kDa, a trimer complex formed by three monomers of SynA 1 + 2 (10.6 kDa). The chemical cross-linking experiment also showed SynA 1 + 2 was in the presence of trimer form (Fig. 2B). CD spectroscopy indicated that SynA 1 + 2 was typical helical with two negative maxima at 208 and 222 nm and high thermo-stable (Fig. 3, A and C).

To further verify the stability of NHR/CHR complex, thermal unfolding was observed by monitoring the changes in ellipticity at 222 nm. The mixture of NHR and CHR in equimolar concentrations, the α-helical content was increased compared with arithmetical sum of spectra of the individual peptides in the absence of an interaction (Fig. 3B). These findings indicate that NHR and CHR can interact with each other and form a highly α-helical conformation. To test whether NHR and CHR can form an oligomeric complex, we checked NHR, CHR, and the mixture of NHR and CHR in Native-PAGE. CHR showed no bands in the gel because it carried positive charges under the native electrophoresis conditions and therefore it migrated out of the gel. NHR showed bands in the gel for its negative charges under the native electrophoresis. The complex showed two bands in the gel. The lower band showed the same position with the isolated NHR and the upper band may be the oligomeric complex of the NHR and CHR (Fig. 4A). To confirm whether the band indeed represents the complex of the NHR and CHR, we analyzed the mixture of NHR and CHR at different ratios by native-PAGE (Fig. 4B). With the increase of CHR, the upper band intensity had been enhanced and the lower band intensity had been decreased, which suggested the formation of the upper band depended on the CHR concentration and indicated that the appearance of the upper band was not the result of the NHR aggregation.

To further verify the stability of NHR/CHR complex, thermal unfolding was observed by monitoring the changes in ellipticity at 222 nm. The mixture of NHR and CHR in equimolar concentration showed partially unfolding, and the increasing temperature steadily reduced the helical content. Comparing the ther-
mal unfolding experimental results of SynA 1 + 2 with that of NHR/CHR mixture, we found that SynA 1 + 2 seemed to be more stable than NHR/CHR (Fig. 3C). To confirm the stability difference between SynA 1 + 2 and NHR/CHR mixture, we did acidic denature and guanidine hydrochloride denature experiments. In the acidic denature experiment, the equilibrium transitions were followed by monitoring the change of $\theta$ at 222 nm upon mixing indicated an interaction between the two peptides increased the total helical content. C, temperature denature at 222 nm for SynA 1 + 2 and NHR/CHR mixture. Final concentration of each peptide was 10 $\mu$M.

FIGURE 3. The secondary structures of SynA 1 + 2, NHR, CHR, and NHR/CHR mixture were assayed by CD spectroscopy. A, CD spectra for NHR (filled triangles), CHR (open triangles), and SynA 1 + 2 (filled circles). Final concentration of each peptide was 50 $\mu$M. B, CD spectra of NHR and CHR before (cross) and after (open square) mixing. The increase in ellipticity at 222 nm upon mixing indicated an interaction between the two peptides increased the total helical content. C, temperature denature at 222 nm for SynA 1 + 2 and NHR/CHR mixture. Final concentration of each peptide was 10 $\mu$M.
ing that they may have an important function. From above analyses, the linker in SynA 1 + 2 may play a vital role in the stabilization of syncytin-A fusion core.

Crystal structure of a protein was essential for understanding the function of this protein well. Because of the lack of high resolution structural data on syncytin-A, we used computational methods to predict the syncytin-A fusion core structure. Based on the sequence similarities analyses, sequence of syncytin-A protein was found to be much more similar with the envelope protein sequences of HTLV, MoMLV, and Ebola, instead of HIV-1 and SIV (Fig. 1A). The reasonable sequence similarities gave us confidence that a homology model could be built on the basis of known x-ray crystal structures of the above envelope proteins (residues 343–402 for HTLV, residues 558–567 for MoMLV and residues 610–630 for Ebola). We chose the shorter sequence (residues 447–528) as model objective due to the lack of crystal coordinates of other sequences. As illustrated in Fig. 6A, the model corresponding to SynA 1 + 2 formed a homotrimer. It disclosed that NHRs formed an inner trimeric structure organized by hydrophobic α-helices association. This inner trimer structure was followed by the predicted disulfide-bonded linker. Next to the linker, an extended domain with a C-terminal helix (residues 513–528) ran anti-parallel into grooves to the central coiled-coil trimer formed by NHRs. The computational molecular contained a buried chloride-binding site (Asn), which was conserved in many other membrane fusion proteins such as MoMLV TM protein and Ebola GP21 core (25, 27).

To further support the above results, we carried out limited proteolysis experiment of the SynA 1 + 2 and identified a protease-resistant core. Digestion of SynA 1 + 2 by proteinase K generated a series of protease-resistant fragments confirmed by mass spectrometry. According to the sequence information from the fragments, we deduced the protease-resistant core: an N-terminal fragment corresponding to residues 513–528 that ran anti-parallel into grooves to the central coiled-coil trimer formed by NHRs. The computational molecular model was in good agreement with our experimental results, and the conservation of this structure in many viral envelope fusion proteins indicated it might be endowed with some crucial biofunctions.

FIGURE 5. Equilibrium isothermal unfolding of SynA 1 + 2 (filled circles) and the mixtures of NHR and CHR with equal molar (open squares) by acid denature and GdnHCl chemical denature. Normalized unfolding curves recorded in PBS at 25 °C with 10 μM monomer concentration are shown. A, acidic denature; B, GdnHCl denature. The fraction folded of proteins \( f^\text{fold} = \frac{\theta - \theta_{\text{random}}}{\theta - \theta_{\text{random}}} \) and \( \theta_{\text{random}} \) were the mean residue ellipticities at 222 nm of the folded state and the unfolded state.
of Dupressoir et al. (17), syncytin-A-mediated cell-cell fusion could be achieved in several different cell lines, especially in 293T cells. With HRA1 peptide of syncytin-1 as negative control (gift from Hungwen Chen, Taiwan) (18), we tested whether our peptides could block syncytin-A-mediated cell-cell fusion in 293T cells transiently expressing syncytin-A. From the results, HRA1 and CHR did not block cell-cell fusion significantly even at concentration 50 μM, while NHR was potently inhibitory. The NHR inhibition IC50 value was about 5.4 μM and completely blocked cell fusion at about 12 μM when the cell fusion efficiency achieved nearly 100% (Fig. 7, A and B). The reason that fusion efficiency was decreased by adding NHR peptides was that they could interfere in the formation of syncytin-A fusion core oligomerization, indicating that syncytin-A fusion core oligomerization was the key factor for cell-cell fusion process.

**DISCUSSION**

Little information is currently available on the fusion mechanism of the newly discovered murine endogenous retrovirus, which encodes highly fusogenic retroviral envelope gene (syncytin-A gene). The only clue about such endogenous retrovirus is that syncytin-A gene is highly placenta-specific expressed and displays fusogenic activities when expressed in transfected cells (17). These features are closely relative to those of human syncytin-1 and syncytin-2 genes, which play a role in trophoblast fusion and placenta morphogenesis (11, 15, 16, 20, 40). We report here a comprehensive study on the structure of the predicted syncytin-A HR regions of murine endogenous retrovirus, which highlights that the HR regions in TM subunit can indeed form a stable complex typical of class I fusion proteins. SynA 1 + 2, including NHR and CHR and a natural linker, forms a high thermo-stable coiled-coil trimer. We also produced two other peptides: a single NHR and a single CHR. We showed that NHR and CHR interacted with each other and formed a stable complex estimated by Native-PAGE and CD experiments. The NHR region itself had a high α-helix conformation and CHR region had a random structure. The high stability of SynA 1 + 2 was similar to that of other viral corresponding regions in envelope proteins. The HIV core has a Tm of 90 °C, SIV core has a Tm > 90 °C, Ebola core has a Tm of 90 °C, SV5 core has a Tm > 90 °C, and HRVS core has a Tm of 90 °C in the presence of 4 M GdnHCl (34, 35, 38, 41, 42). The high stability of the structure formed by SynA 1 + 2 suggests that it represents the stable fusion core of syncytin-A.

On a basis of high similarity of the linkers between N-terminal heptad region and C-terminal heptad region of diverse viral transmembrane regions (Fig. 1A), we believed that the linker might play a vital role in the structural formation and stability. To verify whether the natural linker between NHR and CHR affected the structure of SynA 1 + 2 protein, we compared the stability of SynA 1 + 2 protein containing a single natural loop with NHR/CHR mixture at equimolar concentrations. In the thermal denature experiments, we found that the secondary structure of SynA 1 + 2 remained nearly unchanged from 25 °C to 95 °C, while NHR/CHR mixture partially unfolded in the same temperature range. In the acidic denature assay, the unfolding midpoint for SynA 1 + 2 was pH 4.9, which was lower than that of NHR/CHR (pH 5.6). In the GdnHCl denature experiments, the unfolding midpoints for SynA 1 + 2 and NHR/CHR were 4.8 and 2.5 M respectively, indicating that it needed more GdnHCl to unfold SynA 1 + 2 than NHR/CHR. Taken together, we thought the connective loop linking NHR region to CHR region was required for the stability of syncytin-A fusion core.

We found that the loop in syncytin-A resembled those of MoMLV, HTLV, and Ebola TM proteins. In the MoMLV, HTLV, and Ebola viruses (23, 25, 26, 28), the loop of 5–8 residues forms a well ordered structure at the end of N-terminal helix region and then follows an extended coil of C-terminal region. In SIV gp41, the loop of 26 residues (33) is more mobile than its helical core but still retains identified structure and then follows a long α-helix of C-terminal. Due to the lack of the typical long C-terminal helices as in HIV-1 and SIV gp41 that stabilize the central coiled-coil, this loop structure in syncytin-A may play a vital role in stabilizing intact envelope fusion protein core in a compensative way. The short ordered loops in HTLV, MoMLV, and Ebola viruses may also have the similar compensative function.

The process of trophoblast fusion in the placenta resembles the fusion process between the viral and cell membranes for
viral entry. On the virion surface, the envelope glycoprotein complex exists as a trimeric spike comprising the TM subunit and the surface subunit. The fusogenic syncytin-A protein, originated from the retrovirus 20 million years ago, has a similar structure with other membrane fusion proteins studied earlier, suggesting a uniform fusion mechanism in the trophoblast fusion. It appears that these diverse viruses present fusion peptides to the target cells via a common configuration, in which the fusion peptides are followed by a central, three-stranded coiled-coil that is supported by additional C-terminal structures. Mechanistically, fusion could take place as follows. Fusion peptides insert into the target membrane, and as the fusion proteins bend into six-helix bundles, the fusion peptides and TM domains pull the two membranes locally toward each other (31, 43, 44). Peptides that are derived from the HR regions of retrovirus (37, 39, 45) and paramyxovirus (46, 47) fusion proteins have been shown to strongly interfere with the fusion activities of these peptides. They work in a dominant-negative manner, as the anti-HIV-1 activity of N- and C-peptides (36, 39, 45). It is proposed that C-peptides (C34) may bind to the endogenous N-peptide coiled-coil trimer within HIV-1 gp41, and N-peptides (N36) might interfere with the formation of the central coiled-coil or bind to the endogenous C-peptide regions and then block the formation of fusion-active core of gp41 and inhibit the fusion between the viral and target cell membranes. The understanding of the action of fusion inhibitory peptides launches a new avenue of comprehension of viral fusogenic mechanisms and identification of antiviral peptides against infection by many other enveloped viruses, such as simian immunodeficiency viruses (SIV), respiratory syncytial virus (RSV), Ebola virus, and the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (48–51).

To investigate the functional relationship between the two heptad repeat regions, we tested fusion-inhibitory effect of HR regions of syncytin-A. NHR was capable of inhibiting syncytin-A-mediated fusion in the micromolar range with IC50 concentration about 5.4 μM when cell-cell fusion efficiency achieved nearly 100%; distinct to HIV-1 C-peptides, the mimic CHR of syncytin-A protein in this vital process of pregnancy but also account for that conservation of the virus envelope proteins.

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