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Characterization of BIV Env core: Implication for mechanism of BIV-mediated cell fusion

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Abstract

Entry of lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), requires folding of two heptad repeat regions (HR1 and HR2) of gp41 into a trimer-of-hairpins, which subsequently brings virus and cell membrane into fusion. This motif is a generalized feature of viral fusion proteins and has been exploited in generating antiviral fusion agents. In the present paper, we report structural characters of Env protein from another lentivirus, bovine immunodeficiency virus (BIV), which contributes to a good animal model of HIV. BIV HR1 and HR2 regions are predicted by two different programs and expressed separately or conjointly in *Escherichia coli*. Biochemical and biophysical analyses show that the predicted HRs of BIV Env can form a stable trimer-of-hairpins or six-helix bundle just like that formed by feline immunodeficiency virus Env. Cell fusion assay demonstrates that the HR2 peptide of BIV can efficiently inhibit the virus-mediated cell fusion.

Keywords: Bovine immunodeficiency virus; Feline immunodeficiency virus; Env protein; Heptad repeat region; Six-helix bundle

Biophysical and biochemical studies suggest that the rather diverse enveloped viruses, including retroviruses, paramyxoviruses, filoviruses, coronaviruses, etc., adopt a similar mechanism in membrane fusion [1]. The homologous virus fusion model is based on the similar structures characterized with a trimer-of-hairpins or six-helix bundles, which are formed by two heptad repeat regions (HR1 and HR2) from virus envelope proteins [2]. It was also demonstrated that blocking the trimer-of-hairpins formation by external peptides derived from HR1 or HR2 regions could inhibit virus-cell fusion, which had led to the production of an antiviral drug used in AIDS [3,4]. For the genus lentiviruses of retroviruses, most studies about virus fusion were focused on the well-known HIV and SIV and few works were carried on the other two members, FIV and BIV, especially BIV, named by Gonda in 1987 for its similarity to HIV-1 and SIV in morphology, immunology, and genetics [5,6]. As an infectious retrovirus, BIV induces splenomegaly and lymphademopathy syndromes with associated fatal immune dysfunctions [5]. Although BIV does not appear to infect human, the virus is able to replicate productively at the mucosal surfaces in rabbits, emphasizing the importance of BIV/rabbit system as a good small-animal model [7,8]. Remarkably, BIV-small-animal model can be useful in studying mechanisms of lentivirus persistence as well as methods of intervention relevant to HIV infection, both being crucial in current researches on the fatal disease of AIDS. Unfortunately, despite its potential significance, thus far little is known about BIV, including the structural characters of the envelope protein. It could be imagined

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that BIV might share the same fusion mechanism as HIV and SIV, but there is no evidence to support it yet.

For HIV and SIV, their envelope proteins (Env), required for virus-cell membrane fusion, are synthesized as fusion incompetent precursor, termed gp160, and proteolytically cleaved into two subunits, a surface one (gp120) and a transmembrane one (gp41), activating the fusion potential of the glycoproteins. The gp120 can bind the receptors at cell surface, while the gp41 subunit contains specific domains necessary for virus fusion, including fusion peptide (FP), heptad repeat region 1 (HR1), and heptad repeat region 2 (HR2). Crystal structure indicates the formation of trimer of HR1 and HR2 heterodimers for HIV and SIV [9,10]. As other retroviruses, the surface glycoprotein of BIV is essential to the entry of viral genomes into host cells and genomic analysis of BIV, which suggests that the transmembrane protein is more conservative than the surface protein [5,11], which also indicates that BIV Env might share the common features with homologous proteins in the family of retroviruses, although the fusion peptide of BIV has not been reported up to the present.

In this study, two heptad repeat regions of BIV Env protein were predicted with two different programs. The biochemical and biophysical characters of HR peptides were then revealed by gel-filtration, chemical cross-linking, and CD, etc. As a control, the same work was done on the HR1 and HR2 of FIV Env protein. Results show that HRs of both BIV and FIV can form a stable trimer in vitro. The fusion inhibitory activity of BIV HR2 was also determined.

Materials and methods

Prediction and gene construction. The HR1 and HR2 regions of BIV and FIV Env protein (GenBank Accession Nos. NP_040566 and AAB25466) were predicted by LearnCoil-VMF program (http://nightingale.lcs.mit.edu/cgi-bin/vmf) and the Coils program (http://www.ch.embnet.org/software/COILS_form.html) [12]. The codon usage was optimized according to *Escherichia coli* expression preferences. To get the DNA fragments which encode the 2-Helix peptide containing the linker SGGRGG, a group of overlapping synthesized primers according to the published sequences was designed following the method we described previously [13]. Then the DNAs of HR1 and HR2 were amplified by standard PCR method utilizing the DNA of 2-Helix as template. The genes of 2-Helix, HR1, HR2 with *Bam*HI/*Xho*I sites at the ends were inserted into the *Bam*HI–*Xho*I restriction sites of the GST fusion expression vector pGEX-6p-1 (Pharmacia). The positive plasmids were verified by direct DNA sequencing. The recombinant plasmids were named BIV-2-Helix, BIV-HR1, BIV-HR2, FIV-2-Helix, FIV-HR1, and FIV-HR2, respectively.

Protein expression and purification. Escherichia coli strain BL21 (DE3) transformed with the recombinant pGEX-6p-1 plasmid was grown at 37 °C in 2× YT medium to an optical density of 0.8–1.0 ($OD_{590\,nm}$) before induction with 1 mM IPTG for 4 h. Bacterial cells were harvested and lysed by sonication in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.3; 150 mM NaCl). Triton X-100 was then added to a final concentration of 1%, and the lysates were incubated for 30 min at 0 °C and subsequently clarified by centrifugation at 12,000*g* for 30 min at 4 °C. The clarified supernatants were passed over glutathione–Sepharose 4B column (Pharmacia) that was equalized with PBS. The GST fusion protein-bound column was washed by PBS over 10 column volumes and eluted with reduced glutathione (10 mM) for three column volumes.

The GST fusion proteins were then cleaved by GST-fusion rhinovirus 3C protease (or called GST-3C, kindly provided by Drs. K. Hudson and J. Heath) at 5 °C for 16 h in the cleavage buffer (50 mM Tris–HCl, pH 7.0; 150 mM NaCl; 1 mM DTT; and 1 mM EDTA, pH 8.0). GST-3C protease recognizes the same cleavage site for the GST fusion protein as that of the commercial PreScissionTM (Pharmacia). The free GST, or non-cleaved GST-2-Helix and GST-3C were removed by passing over the glutathione–Sepharose 4B column again. The resultant proteins were dialyzed against PBS before being concentrated to a proper concentration by ultrafiltration, and stored at −70 °C for further analysis. Proteins were analyzed on Tris–Tricine SDS–PAGE and the protein concentration was determined by the BCA assay (Pierce Biochemicals).

Mass spectrometry. The purified protein in buffer of 20 mM Tris–HCl, pH 8.0 was analyzed by using Bruker Daltonics Biflex III MALDI-TOF mass spectrometer.

Gel-filtration analysis. The GST-removed purified 2-Helix protein was loaded on the Hiload Superdex TM G75 (Amersham-Pharmacia) column with Akta Explorer FPLC system (Amersham-Pharmacia). The fractions of the peak were collected and run on Tris–Tricine SDS–PAGE. The peak molecular weight was estimated by comparison with the protein standards run on the same column.

Chemical cross-linking. The purified 2-Helix protein was dialyzed against cross-linking buffer (50 mM Hepes, pH 8.3; 100 mM NaCl) and concentrated to about 2 mg/ml by ultrafiltration (10 kDa cut-off). Proteins were cross-linked with ethylene glycol bis(succinimidylsuccinate) (EGS) (ICN). The reactions were incubated for 1 h on ice at concentrations of 0, 0.1, 0.5, 2.0, 4.0, and 6.0 mM EGS, respectively, and stopped by the addition of 50 mM glycine amide. Cross-linked products were analyzed under reducing conditions on 14% SDS–PAGE.

CD spectroscopic analysis. CD spectra were recorded on a Jasco J-715 spectrophotometer with proteins in PBS buffer (10 mM sodium phosphate, pH 7.3; 150 mM NaCl) at 200–250 nm. Wavelength spectra were recorded at 25 °C using a 0.2-cm pathlength cuvette. Each spectrum was obtained by averaging five scans at a scan speed of 200 nm/min with a step resolution of 0.5 nm. Thermodynamic stability spectrum was recorded from 25 to 80 °C at five points (25, 0, 70, 80, and 85 °C).

Cell fusion assay. Fetal bovine lung (FBL) cells were used to propagate the BIV strain R29 in these experiments. The FBL cells were plated in a 24-well tissue culture plate at a density of 5×10^4 cells per well in DMEM. The following day, monolayers of FBL cells with or without a series of protein preparations were infected with 1:5 dilution of BIV R29 strain (cocultured with FBL cells at a density of 1×10^4 cells) for 3 h at 37 °C. Then the inoculun was removed and DMEM (2% FCS, 0.1% P/S) was added to the cells. The cells were monitored daily for syncytium formation and the syncytia were observed at 200× magnification after 48 h incubation at 37 °C in 5% CO_2 incubator. At least four random different fields of inverted microscope were counted and recorded at the percentage of nuclei numbers in polykaryons to numbers of total nuclei. Cell fusion assay was performed with high-purified GST, GST-2-Helix, and GST-HR2.

Results and discussion

Heptad repeat regions prediction

Two different programs, LearnCoil-VMF and Coils, were employed to predict the heptad repeat regions for the BIV Env protein. As illustrated in the research work

of Kim and co-workers [12], LearnCoil-VMF is a specialized program for identifying the coiled-coil-like regions that compose the trimer-of-hairpins motif in viral membrane fusion proteins. Results show that this program is able to predict two such regions (corresponding to HR1 and HR2) in almost all viral membrane fusion proteins, including retrovirus envelope proteins, paramyxovirus fusion proteins, orthomyxovirus hemagglutinins, coronavirus spike proteins, arenavirus glycoproteins, and baculovirus envelope glycoproteins. Most of these predictions have been supported by the crystal structures [9,14–16,10,17,18]. In the lentiviruses, two HR regions were successfully predicted by LearnCoil-VMF in the envelope proteins of HIV-1, SIV, FIV, Visna virus, and CAEV. Instead, only one HR region was detected in the Env protein from BIV with the same program (Fig. 1A). However, another completely different region can be predicted with high likelihood in BIV Env by the Coils program (Fig. 1A). These two predicted regions are located in the similar positions of HR1 and HR2, which occur also in the Envs of other lentiviruses, like HIV and FIV (Fig. 1C). Therefore, it might be suggested that the two regions predicted by LearnCoil-VMF and Coils were corresponding to HR1 and HR2, respectively (Figs. 1A and B). The amino acid sequence multiple alignments also indicate the similar characters of the possible HR1 and HR2 of BIV Env as those of other lentiviruses, in which the residues a, d in the heptad repeats are almost all hydrophobic amino acids (Figs. 2A and B).

The GST fusion BIV or FIV HR1, HR2, and 2-Helix were all expressed as soluble proteins at 37 °C (for HR2) or 25 °C (for HR1 and 2-Helix)

As described under Materials and methods, all the constructs were synthesized by PCR and expressed as GST-fusion proteins in *E. coli*. The GST-fusion HR2s both from BIV and FIV were expressed as soluble proteins at 37 °C, while GST-fusion HR1s and 2-Helixes were only partly soluble when expressed at 25 °C. The purified GST-fusion and untagged HR1s have a tendency to aggregate and cannot be concentrated to a high concentration, probably due to the high hydrophobicity. The HR2s and 2-Helixes can be easily purified by GST affinity column with a high yield. MALDI-TOF mass spectrometry analysis showed that the molecular weights of BIV-HR1, BIV-HR2, FIV-HR1, and FIV-HR2 were 6.9, 3.5, 7.1, and 4.7 kDa, respectively, similar as the calculated ones (data not shown).

2-Helix proteins could form trimers in vitro

Gel-filtration analysis showed that the 2-Helix proteins of BIV were eluted between positions corresponding to protein standards 52 and 14.4 kDa (Fig. 3), while the predicted molecular weight was about 9.5 kDa. This observation might indicate that the 2-Helix protein could form polymers. Further analysis by chemical cross-linking confirmed the trimer forma-

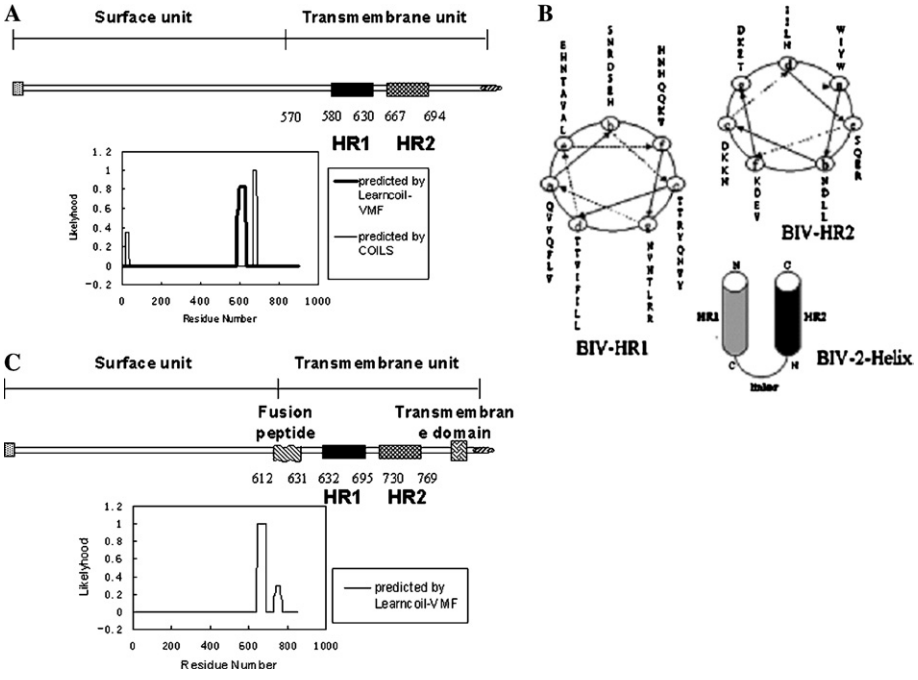

Fig. 1. Prediction of the heptad regions of BIV and FIV Env proteins using the program LearnCoil-VMF and COILS. (A) Schematic diagram of BIV Env protein with the location of structurally significant domains as indicated. (B) Helix wheel analysis of the predicted coiled-coil regions of BIV HR1and HR2 as indicated. (C) Schematic diagram of FIV Env protein with the location of structurally significant domains as indicated.

A The lentivirus HR1 (N-terminal coiled coils) alignment

```
          abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefga
HIV-1     ---------------SGIVHQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL------------
SIV       ---------------AGIVQQQQQLLDVVKRQQELLRLTVWGTKNLQTRVT------------
CAEV      ----------TKAAVQTLANATAAQQDVLEATYAMVQHVAKGVRILEARVARVEA--------
EIAV      ---------------WHTFEVENSTLNGMDLIERQIKILYAMILQTHADVQLLKERQQ-----
Visna     ---------------QSLANATAAQQNVLEATYAMVQHVAKGVRILEARVA------------
FIV       GATAIGMVTQYHQVLATQQEAIEKVTEALKITNLRLVTLEHQVLVIGLKVEAMEKFLYTAFAMQ
BIV       --TTLVNQHTTAKVVERVVQNVSYIAQTQDQFTHLFRNINNRLNVLHHRVSYLE----------
```

B The lentivirus HR2 (C-terminal coiled coils) alignment

```
          abcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
HIV-1     -------WMEWDREINNYTSLIHSLIEESQNQQEENEQELL-
SIV       -------WQEWERKVDFLEENITALLEEAQIQQEKNMYELQ-
CAEV      ------TWQQWERGLQGYDTNLTILLKESAAMTQLAEEQ---
EIAV      HLNESTQWDDWVSKMEDLNQEILTTLHGARNNLAQSMITFN-
Visna     -------WQQWEEEIEQHEANLSQLLREAALQVHIAQRDAQ-
FIV       -----ITLGEWYNQTKDLQKKFYGIIMDIEQNNVQGLQQLQK
BIV       ---NSKTWNDLQDEYDKIEEKILKIRVDWLN-----------
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Fig. 2. The multiple alignments of HR1 (A) and HR2 (B) of seven typical lentiviruses (HIV-1, SIV, CAEV, EIAV, Visna, FIV, and BIV).

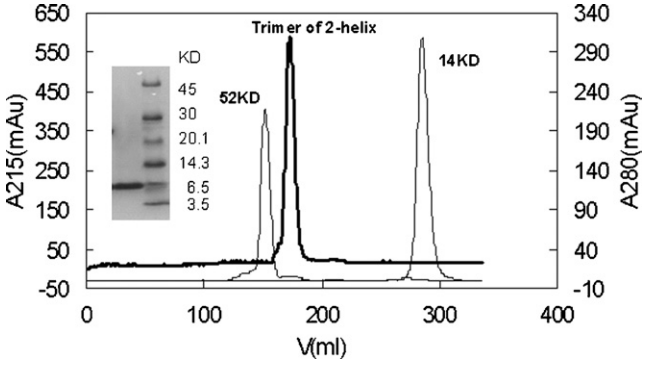

Fig. 3. Gel-filtration analysis of BIV 2-Helix. 2-Helix proteins run on Superdex G75 gel filtration. Inset was proteins from the peak run on Tris–Tricine SDS–PAGE. The 2-Helix protein was eluted from the column between the eluted volumes corresponding to 52 and 14.4 kDa protein standards, which demonstrated that the 2-Helix protein could form polymer.

tion of 2-Helix (Fig. 4A). Instead of the natural connecting sequence, linker SGGRGG is utilized to connect HR1 and HR2, omitting the disulfide bond present in the natural state. The resulting trimer structure of 2-Helix shows that the interaction between HR1 and HR2 in BIV is most probably an intrinsic tendency, demanding no external impact such as a covalent bond. This is also true in other lentiviruses. The same results were also obtained with FIV 2-Helix protein (Fig. 4B). These results provide the evidence that the predicted HR regions of BIV Env protein are similar in characters to those in other lentiviruses, although only one HR was predicted by LearnCoil program.

The complexes of HR1 and HR2 are stable α-helix bundles

Far-UV CD spectropolarimetry was employed to analyze the secondary structures of proteins. The CD spectra showed that both BIV and FIV 2-helixes had minima at 208 and 222 nm, consistent with a typical α-helix structure (Fig. 5A). The CD spectra of HR1s also indicate the characteristic of α-helix, while HR2 appeared to be largely random coils characterized by a minimum around 200 nm. However, the mixture of HR1 and HR2 in equimolar amounts at room temperature for 1 h exhibited a CD spectrum curve with intensified double minima at 208 and 222 nm compared to HR1 or HR2 alone, which indicates a higher α-helix content, demonstrating the helical interaction of HR1 and HR2 (Figs. 5C and D). Thermodynamic stability analysis also showed that the 2-Helix protein was thermal-stable with a T_m as high as 80 °C (Fig. 5B). All these results provide the evidence that the predicted HR1 and HR2 peptides of BIV can form a stable six-helix bundle or a trimer of HR1/HR2 heterodimers, which might represent the core structure of Env protein at the postfusion state. This core structure has been well studied in the family of lentiviruses as well as many other enveloped viruses. Therefore, BIV might share the same fusion mechanism as the other lentiviruses and the HR peptides could also be inhibitors of virus fusion.

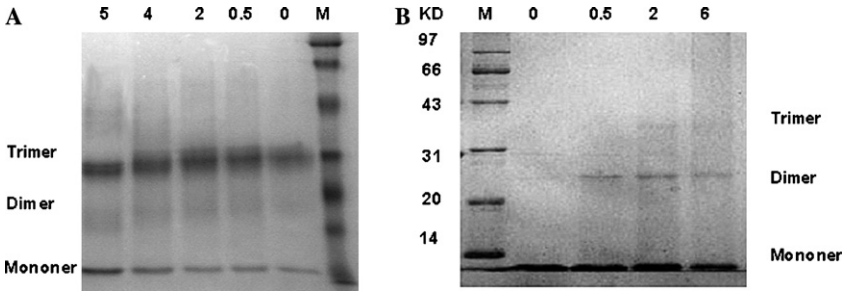

Fig. 4. Chemical cross-linking of 2-Helix proteins. Cross-linking products were separated on 14% SDS–PAGE followed by Coomassie brilliant blue staining. Protein markers are shown in kilodaltons. The numbers over the picture indicate the concentration of ethylene glycol bis(succinimidyl succinate) (EGS) in millimolar used. Bands corresponding to monomer, dimer, and trimer are indicated. (A) BIV 2-Helix; (B) FIV 2-Helix.

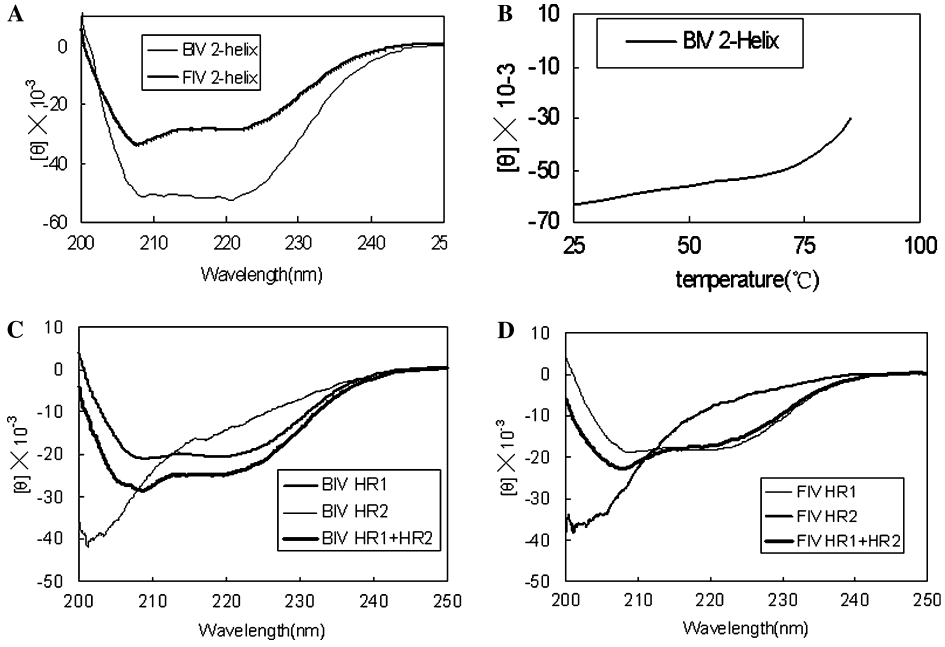

Fig. 5. CD spectra of HR1, HR2, mixtures of HR1 and HR2, and 2-Helix polypeptides. (A) CD spectra of BIV and FIV 2-Helix show a typical α-helix secondary structure with double minima at 208 and 222 nm. (B) Thermodynamic stability spectrum of BIV 2-Helix shows that the T_m is 80 °C. (C) The BIV HR1 and mixtures of two peptides show α-helix secondary structure while HR2 peptide appears largely unfolded. Mixtures of two peptides (HR1 + HR2) show a typical high content of α-helix secondary structure than HR1 and HR2 peptides individually. (D) The FIV is same as the BIV in (C).

BIV HR2 peptides could be inhibitors of virus-mediated cell fusion

The results above demonstrate that the predicted BIV HR regions have the common characters as those of HIV or FIV. It is also interesting to determine the fusion inhibition activity of the peptides, which had been confirmed on HIV [19] and FIV [20]. In the tests of cell fusion inhibition, syncytium formations were actually inhibited by the treatment of GST-HR2, while syncytia were not affected in the cell monolayers tested with GST and GST-2-Helix (Fig. 6). According to our previous research on Newcastle disease virus (NDV), the fusion partner GST (26 kDa) does not affect the inhibition [21]. The non-inhibition of GST-2-Helix indicates that HR2 peptides work as competitors to the endogenous HR2 regions by binding to the HR1 sites of Env. The IC_{50} value of HR2 was measured to be 1.17 ± 0.3 μM. This result shows again that BIV shares the similar fusion mechanism as HIV. For enveloped virus, it has been shown that, unlike the HR2 regions, not all HR1s show fusion inhibitory effects [19,21]. More importantly, in our system the GST fusion HR2 showed an inhibitory effect similar to that of free HR2s in other viruses [19].

Our biophysical and biochemical data strongly support the idea that the two coiled-coil regions predicted by two separate programs, LearnCoil-VMF and Coils, represent the two heptad repeat regions of BIV Env, which are involved in trimer-of-hairpins formation dur-

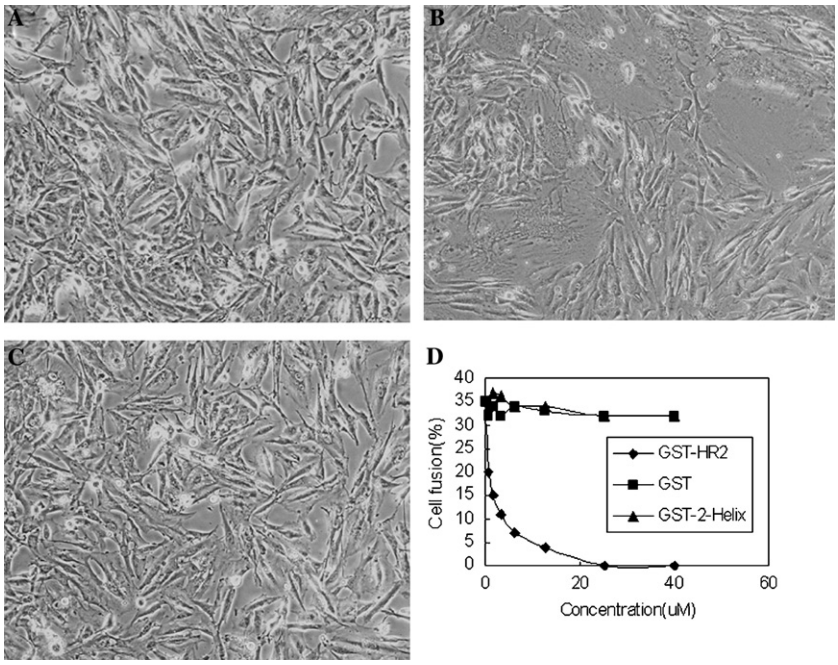

Fig. 6. Fusion inhibition test of the purified proteins. (A) Normal FBL cells. (B) BIV R29 strain infected FBL cells, forming typical syncytia. (C) BIV GST-HR2 incubated with R29 infected FBL cells. It is clear that the formation of the syncytia was inhibited. (D) Inhibition curves of cell fusion by BIV GST, GST-2-Helix, and GST-HR2. Data show that IC_{50} of GST-HR2 is 1.17 ± 0.3 μM.

ing virus fusion as suggested in other enveloped viruses. The HR1 and HR2 peptides from BIV show exactly the same interacting activities as those shown by corresponding regions in other lentiviruses, which indicates the probability of similar roles in virus-cell fusion. This is another evidence, which demonstrates that all the envelope proteins from different lentiviruses have common structure features, despite the lack of sequence homology among them. Thereby, the same fusion mechanism based on structural similarities is shared by these viruses, and a universal antiviral strategy could be used.

BIV HR2 shows a strong virus-cell fusion inhibitory activity based on the interaction of HR1 and HR2, just like situations in HIV. In HIV-1, the C-peptides (derived from HR2), effective at nanomolar concentrations, are much more potent than N-peptides (derived from HR1), which require micromolar concentrations for effectiveness. The C-peptides could inhibit entry by competitively binding to the conserved groove on surface of the endogenous HR1 central coiled-coil trimer, which is also the binding site of outer C helices within viral gp41. The N-peptide might inhibit fusion by interfering with formation of the central, coiled-coil trimer within viral gp41 and/or by binding to endogenous viral HR2 region [9]. The broad inhibitory activity of C peptides against diverse HIV isolates is explained by the highly conserved hydrophobic groove to which these peptides bind. Toward the bottom of the groove is a deep cavity that is filled by three hydrophobic residues from the C helix. This pocket is potentially a good target for inhibiting HIV invasion [1]. Our study shows that HR2 in BIV has a strong inhibitory activity against virus-cell fusion.

Our data also suggest that BIV could also be used as a model of HIV to study the mechanism of virus fusion and inhibition, just as the FIV/cat system [22]. BIV and FIV could share the same fusion mechanism with HIV-1 and SIV based on structural similarities. The crystal structures of the trimer of HR1/HR2 heterodimers in HIV-1, SIV, and Visna virus of lentiviruses, as well as Ebola of filoviruses, SV5 and HRSV of paramyxoviruses, and mouse hepatitis virus of coronaviruses, indicate that three HR1 domains are parallel coiled-coil in the interior of the bundle, and three HR2 domains are packed in an antiparallel manner in the grooves of the HR1 trimer. It will be helpful for fusion inhibition study to determine the interaction characters of HR1 and HR2 from BIV and FIV at molecular level, which is currently under our effort.

Acknowledgments

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