Important Changes in Biochemical Properties and Function of Mutated LLP12 Domain of HIV-1 gp41

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The human immunodeficiency virus type 1 gp41 possesses an unusually long and conserved cytoplasmic region. Mutations in the LLP12 domain in this region have been shown to significantly affect viral competence. It is likely that the impaired infectivity of this mutated virus involves certain biochemical aspects of the peptide LLP12. To test our assumptions, some important biochemical properties and functions of LLP12 domain were studied. The recombinant peptide LLP12 (LLP12 domain on gp41, including LLP1 and LLP2 domains) was prepared via bacterial expression system. Biochemical analysis directly demonstrated its multimeric potential and membrane-binding ability. Several arginine residues in this domain were observed to be extremely highly conserved. Interestingly, the LLP12 mutants constructed by substitution of these arginine residues with alanine (separate mutations in LLP1 or LLP2 or both) showed apparent decreases in their multimeric potential and membrane-binding ability. Comparing our results with independent data on human immunodeficiency virus from other researchers, it appears that both the multimeric state and the membrane affinity of the LLP12 domain of human immunodeficiency virus type 1 gp41 could be involved in viral competence and in the mechanism of human immunodeficiency virus type 1 Env-mediated cell fusion.

Key words: human immunodeficiency virus type 1 gp41, LLP12 domain, membranotropic, multimerization, point mutation

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The cytoplasmic domain of human immunodeficiency virus type 1 (HIV-1) envelope (Env) transmembrane (TM) glycoprotein gp41 plays important roles in the virus life cycle. The existence of its unusually long endodomain, more than 150 amino acids, when compared to the 20–40 amino acids for most other retroviruses, suggests its own biological significance, although not fully understood. Recently, great interest has grown in this domain, especially with respect to two highly conserved cationic amphipathic α-helical motifs, known as lentivirus lytic peptide 1 and 2 (LLP1 and LLP2). It is implied by increasing evidence that various functions, including virus replication, infectivity, transmission, and cytopathogenicity, are closely related to the cytoplasmic domain (1–5). The functions of the cytoplasmic domain have been examined by creating frameshifts, deletions, or truncations, indicating that this domain can affect viral replication, infectivity, and cytopathicity (1–5). Mutant HIV lacking the Env cytoplasmic domain cannot replicate in peripheral blood mononuclear cells and in the majority of T cell lines in vitro (6). Some point mutations in gp41 endodomain reduce apoptosis and calmodulin binding without affecting viral replication (7). Point mutations in LLP2 domain result in decreased binding of neutralizing antibodies to the Env ectodomain (8). Interestingly, Kalia and colleagues reported that arginine substitutions in LLP12 could affect the virus infectivity in a single-cycle assay (9). An explanation for all of these phenomena requires knowledge of the exact structure and function of the cytoplasmic domain of gp41; however, this has not been demonstrated directly.

To reveal the important role of Env cytoplasmic domain in virus infection, there are increasing research on the properties of this region, especially the LLP12 domain (comprising both LLP1 and LLP2 domain). Circular dichroism (CD) studies of synthetic LLP1 and LLP2 peptides showed that their α-helicity increased with the addition of lipids, indicating the formation of lipid-associating amphiphatic helices (10,11). A recombinant fusion protein of gp41 cytoplasmic tail fragment and an Escherichia coli beta-galactosidase was expressed in living mammalian cells to examine the membrane-binding ability of this region (12). Moreover, two 15-mer gp41-derived peptide libraries, covering the entire endodomain, were constructed and detected for their membranotropic (membrane-binding) properties (13). To prove that the cytoplasmic domain has higher-order structures, a fusion protein of the gp41 cytoplasmic domain and the E. coli malE protein was generated and demonstrated to have a tendency to self-assemble into a multimeric complex, which, in turn, may play a key role in the virus life cycle (14).

With the evidence that some site-direct arginine mutants showed decrease in infectivity and defect in replication or impairment in inducing syncytium in T-cells lines (9), the importance of arginine residues in LLP12 is indicated. Nevertheless, the reason why changing only a few amino acids exerts such a severe influence on such
virological properties has not yet been explained. Based on an interest to characterize the effects that mutations in LLP12 have on the structural and functional properties of this important viral protein, we sought possible evidence for a correlation with mutant’s decreased infectivity, and we mutated several highly conserved arginine residues within the LLP12 domains, and examined their multimeric potential and membrane-binding ability in this study. Comparing our results with previous virus experiments by others researchers (9), it appears that the aforementioned biochemical properties of LLP12 domain of HIV-1 gp41 may be involved in viral infectivity and the HIV-1 Env-mediated cell fusion.

Materials and Methods

Cell lines and transient transfection

293T and HeLa cells were grown in Dulbecco’s minimum essential medium (Pierce, Rockford, IL, USA) complemented medium supplemented with antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin) and 10% fetal bovine serum (FBS; Pierce). Transfection was performed using Vigofect reagent (Vigorous Biotechnology, Inc., Beijing, China) according to the manufacturer’s protocol. Cells were plated in six-well tissue culture plate 1 day before transfection. Cells at 40–60% degree of confluency were transfected with 5 μg of plasmid DNA. Cells were collected 24 h after transfection.

Mutagenesis and construction of plasmids

Single-point substitutions as illustrated in Figure 1A were introduced into the gp41 cytoplasmic tail-coding sequence of the HXB2 by oligonucleotide-directed, site-specific mutagenesis using a PCR overlap extension. For each type of mutations to be generated (affecting arginine residues 851 and 853 in LLP1 and arginine residues 775, 777 and 793 in LLP2), two pairs of primers [EcoRI/BamHI forward primer (p1) and XhoI reverse primer (p2) and mutagenic forward primer (p3) and mutagenic reverse primer (p4)] were used. All these primers designed to encode arginine-to-alanine changes were as follows: LLPM1 (R851A and R853A), 5'-GATAATTCAGATCTACCCCGCTTTGAG-3' (p1) and 5'-CTGACTCGATTATGCAAATCCTTCCC-3' (p2) and 5'-CATACCTAGAGCAAATGACAGGCTTTG-3' (p3) and 5'-CCAAGCCCTGTGCTATTGCTAGATGATG-3' (p4). LLPM2 (R775A, R777A, and R793A), 5'-AATTGGATCCTTACTACCACGCTGCTCTCGACTTCTTG-3' (p1), 5'-CTGACTCGATTATGCAAATCCTTCCC-3' (p2), 5'-CTCCCCACCAGCCGGTCTCCAG-3' (p3), 5'-CTTTGGAGCCCGGGGTGGAAG-3' (p4).

In the first round of PCR, two amplification reactions were performed, both using the gp160 clone as the template. One amplification used oligonucleotides p1 and p4, and the other used was p2 and p3. The two PCR products were then amplified in a second round of PCR, by using p1 and p2 as primers, to generate a 300-bp fragment of LLPM1 and LLPM2 containing the desired mutations in

Figure 1: Construction diagram of Human immunodeficiency virus type 1 (HIV-1) LLP12 point substitution mutants and the numbers of arginine residues in different viruses’ cytoplasmic domains. (A) The corresponding conservations of mutated arginine residues. The amino acid sequence in single letter indicates the residues 773–793 and 833–861 located in the C-terminus of the HIV-1 gp41 cytoplasmic tail of wild-type (WT; 9). The conservation of mutated arginine residues among 1103 different HIV gp41 sequences from National Center for Biotechnology Information are shown at the top. (B) The numbers of arginine residues in cytoplasmic domains of 14 different viruses. According to the length of these domains from short to long, viruses are arranged from left to right in this diagram. These cytoplasmic protein sequences come from Human Influenza H3N1 Virus Hemagglutinin2 (HA2), SER virus F protein (SER F), Human Respiratory Syncytial Virus F protein (H RSV F), Vesicular Stomatitis Virus (VSV), Newcastle Disease Virus (NDV), Human para-influenza virus type I (HPIV1), Sandia Virus F Protein (SV), Human Coronavirus Strain 229E (H229E), Canine Coronavirus Virus (CCV), Transmissible Gastroenteritis Virus (TGEV), Bovine Coronavirus Virus (BCV), Influenza A virus M2 protein (IV M2), Human Herpes Virus (HHV), and HIV-1 LLP12 domain.
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LLP1 and LLP2 domains. Then LLP1 was used as the template to generate the mutant of LLPM3, which contains both mutations in LLP1 and LLP2 domains. All the WT and mutated DNA fragments were ligated into the expression plasmid pGEM-6p-1 via its BamHI and XhoI restriction sites, and also into the transfection plasmid pCDNA3 via its EcoRI and XhoI restriction sites.

Expression and purification of recombinant proteins

The pGEM-6p constructs were used to transform E. coli strain Rosetta. GST fusion proteins were isopropylthiogalacto-pyranoside induced and overexpressed for 2.5 h at 37 °C. The cells were lysed with a lysis buffer (1% Triton-X-100, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride) using sonication. After centrifugation at 27 215 g at 4 °C for 20 min, the supernatants containing the fusion protein were clarified and transferred to prepared glutathione resin (Glutathione-Sepharose 4B affinity column). After washing resin with ordinary wash buffer, 50 mM reduced glutathione (pH 8.0) could be used as additional wash buffer to remove GST protein from resin, and the GST−LLP12 fusion proteins were still retained in the resin because of the unique nature of LLP12 peptide. The GST−LLP12, GST−LLPM1, GST−LLPM2, GST−LLPM3 fusion proteins were then eluted, with special elution buffer containing both 50 mM reduced glutathione (pH 8.0) and 0.05% Triton-X-100 (v/v). On the other hand, GST−LLP12 fusion proteins could be cleaved by PreScission Protease on the resin. Similarly, cleaved GST were first washed out with 50 mM reduced glutathione (pH 8.0), and LLP12, LLPM1, LLPM2, and LLPM3 were finally eluted with Tris–HCl buffer containing 0.05% Triton-X-100 (v/v; pH 8.0). All the purified proteins were analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) under reducing conditions.

Circular dichroism spectroscopy

Circular dichroism spectra of these purified proteins, including LLP12, LLPM1, LLPM2, LLPM3 in water (pH 7.4), were obtained using an Aviv 190 spectropolarimeter (Ultrospec 4300 pro, Pharmcia, USA) by averaging three measurements. Water containing 0.05% Triton-X-100 (v/v) was used as the proper control. Spectra were scanned in a capped quartz optical cell with a 1 mm path length, at 25 °C.

Antibodies and ELISA

Peroxidase-conjugated rabbit anti-mouse immunoglobulins were obtained from Dako (Dako, Glostrup, Denmark). Mice were immunized subcutaneously with 30 μg/g purified LLP12 per mouse in complete Freund’s adjuvant (1:1 ratio) at a final volume of 200 μL. Boosters were given in incomplete Freund’s adjuvant on days 14 and 28. Sera were separated 7 days after the last boosting immunization. Immunized sera were collected before immunization and used as normal serum. The antibodies in mouse antisera were detected in an enzyme-linked immunosorbent assay (ELISA). The proteins, including LLP12, LLPM1, LLPM2, and LLPM3 (5 μg/mL), were coated overnight in a microtiter plate at 4 °C. A total of 0.25% gelatin on Tris-buffered saline (TBS) was used for more than 2 h to block non-specific binding. After washing twice with TBS containing 0.5% Tween-20, mouse sera at different dilutions were added and incubated for 1 h at room temperature. After washing (three times) peroxidase-conjugated rabbit anti-mouse immunoglobulins were added. After an additional five washes with 3 min per wash, freshly prepared α-phenylene-diamine dihydrochloride peroxide solution (Sigma, St Louis, MO, USA) was added and the optical density (OD) was measured with microtitrre plate reader (Bio-Rad, USA) at 450 nm.

Membrane-binding ability assay by flow cytometry

293T cells (1 × 10⁶) were incubated with purified proteins for 1 h in a 50 μL final volume, including LLP12, LLPM1, LLPM2, and LLPM3 (25 μg/mL, TBS buffer with 2% FBS). After washed with TBS containing 2% FBS three times, cells were incubated with mouse antisera (1:1000) prepared in advance for 45 min in 50 μL volume. The cells were washed again three times, then they were incubated with 50 μL FITC-conjugated rabbit anti-mouse IgG (Dako, 1:40 dilution in TBS containing 2% FBS) for 45 min. The cells were washed again three times, and then gently resuspended in 500 μL final volume of TBS. Cells were analyzed on the FACSCallibur Flow Cytometer (Becton-Dickinson, Billerica, MA, USA).

Size exclusion chromatography

Purified proteins or molecular mass markers were fractionated by gel filtration through a Superdex 200HR 10/30 column (Amersham Bioscience, Piscataway, NJ, USA) and eluted with TBS containing 0.05% Triton-X-100 (v/v; pH 8.0). The column was connected with an Fast Protein Liquid Chromatography (FPLC) system (Amersham Biotech, Piscataway, NJ, USA) equipped with a UV monitor at a flow rate of 0.5 mL/min at room temperature. Collected samples were analyzed by Western blotting using mouse antisera to identify the proteins.

Results

Bioinformatics analysis of LLP12 domain

The mutations were designed in the LLP1 and LLP2 domains, respectively, or in combination to evaluate the significance of conserved arginine residues for the structural and functional properties of LLP12 domain (residues 768–855), with a similar experimental design of previously reported studies (9). Based on ClustalX (15) analysis among over one thousand different HIV gp41 sequences from National Center for Biotechnology Information (NCBI), some positively charged arginine residues are highly conserved (>74% conservation in LLP2 and >97% in LLP1; Figure 1A). Also, cytoplasmic domains of 13 different viruses selected randomly from NCBI were compared with LLP12. This comparison indicated that the highest frequency of arginine residues was found in LLP12 (13.5%) of HIV-1 (Figure 1B). Based on these analyses, three mutants were constructed with substitutions of arginine to alanine in either the LLP1 (LLPM1) or the LLP2 (LLPM2) or in both domains (LLPM3) to study the effects of certain conserved arginine residues for the structure and function of LLP12 domain.

Secondary structure changes of LLP12 mutants

The prepared and purified LLP12 and mutants exhibited bands with a molecular weight of about 10 kDa and a few polymers
with different size on SDS-PAGE gel, corresponding to the predicted molecular weight and characterization of LLP12 with its mutants (Figure 2). This series of peptides were difficult to purify using conventional methods relative to their hydrophobic properties, therefore we modified the protocol and solution formulation after repeated experiments (see Materials and methods, Figure 2A). Even in the reduced SDS-PAGE, some LLP12 peptide oligomeric forms could also be observed (Figure 2B), with similar results to previous studies (14).

To investigate the changes in their secondary structures, the wild type and mutant LLP12 proteins were detected by CD. Surprisingly, substitutions of only a few arginines lead to distinct structural alterations. As illustrated in Figure 3A, a substantial change in the CD spectrum was observed for LLP12 mutants in comparison to the wild type. A more significant change in the spectrum was observed for LLPM3 mutants. The large positive increase in ellipticity at 222 nm is consistent with a structure change from $\alpha$-helix to random coil.

Identification of multimerization potential of LLP12 and mutants

The multimerization potential of LLP12 and mutants was investigated by FPLC using a Superdex 200HR 10/30 column (gel filtration). During gel filtration chromatography, larger molecules cannot enter the gel's pores; consequently, larger molecules could be eluted faster. When the purified LLP12 protein (wild type) was eluted at 12 min, it was shown that LLP12 was prone to self-assemble into a complex over 150 kDa (Figure 3B). In comparison to LLP12, the fractionation peak of LLPM1 was observed about 1 min later. Therefore, the mutations in LLP1 domain demonstrably decreased the multimerization potential of LLP12 protein. However, the LLPM2 mutant was eluted almost at the same time as the wild type, suggesting that the mutants in LLP2 domain did not affect the multimerization formation of the whole protein (LLP12). Then LLPM3 mutant was eluted almost at the same time as LLPM1, which could be explained based on the previous results. The eluted proteins at each peak were collected and tested by Western blotting (Figure 3B), showing that these were indeed LLP12 peptides (about 10 kDa).

Identification of cellular membrane-binding ability of LLP12 and mutants

Another crucial reported function of LLP12 domain is the membrane-binding ability (12), which was also significantly affected by arginine substitutions in our experiments. In comparison with the LLP12 wild type, all LLP12 mutants showed an impaired ability in binding to the outer membrane of HeLa cells relative to flow cytometry analysis (Figure 4A–D). The binding affinities of the mouse antisera and all four involved proteins, together with GST as the proper control, were proved in ELISA (Figure 4E). To further test the changes in intermembrane-binding ability in vivo, LLP12 fused with EGFP was inserted into pCDNA3 plasmid, which was then transfected into 293T cells. These cells were examined under fluorescence microscope 24 h later (Figure 4F). There were intense fluorescence signals in the perinuclear area of EGFP-LLP12 in contrast to EGFP control, and this confirmed previous research studies on LLP12 intermembrane-binding ability (12). A similar phenomenon was observed in cells transfected with EGFP-LLPM1, EGFP-LLPM2, and EGFP-LLPM3, indicating that none of these mutants notably affected their intermembrane targeting signal on LLP12. A possibility exists that other membrane or scaffold proteins were involved in this membrane-binding process, as exemplified by the calmodulin system (7,16–18).
Kalia and colleagues reported that the HIV-1 viruses having some arginine residues mutated in the LLP1 domain on gp41 were replication defective and showed an average of 85% decreased infectivity as well as that such LLP2 mutants were impaired in the ability to induce syncytia in T-cell lines (9). Based on the interest to find the possible explanation for the decreased infectivity in these

Figure 3: (A) Secondary structural changes detected by CD spectroscopy. Circular dichroism spectra for wild type and LLP12 mutants of 300 µM at 25 °C were showed by molar ellipticity versus wavelength. (B) Fast protein liquid chromatographic analysis (gel filtration chromatography) of LLP12 and mutants. Ba, LLP12 (solid line, 0) and LLPM1 (dotted line, I); Bb, LLP12 (solid line, 0) and LLPM2 (dotted line, II); Bc, LLP12 (solid line, 0) and LLPM3 (dotted line, III). Purified proteins was injected into a Superdex 200HR 10/30 size exclusion column and eluted with Tris-buffered saline containing 0.05% Triton-X-100 (pH 8.0). The protein elution pattern was measured by UV absorption at 280 nm. The elution peak of IgG (150 kDa), which was taken as a molecular mass standard, is marked at the top of the image with arrows. Bd, all the elution samples (the concentration of Triton-X-100 was adjusted to 1% before sodium dodecyl sulfate polyacrylamide gel) were analyzed by Western blotting using antisera.

Discussion

Kalia and colleagues reported that the HIV-1 viruses having some arginine residues mutated in the LLP1 domain on gp41 were
mutated viruses, we examined the multimeric potential and membrane-binding ability of LLP12 and its mutants in this study. We assumed that the mutations were likely to change the biochemical properties of the peptide LLP12, and that these would correlate with the nature of entire membrane protein gp41 and the viral infectivity. To test these assumptions, the peptide and several of its mutants were purified and studied in various ways.

First, bioinformatic analysis confirmed that several arginine residues in the LLP12 domain are highly conserved (Figure 1A). Besides, cytoplasmic domains of 13 different viruses selected randomly from NCBI were compared with LLP12 to show relative highly arginine residues proportion in LLP12 (Figure 1B), indicating that high conservation and high proportion of arginine residues in LLP12 is a typical structural property of HIV-1 gp41. Based on these analyses, three
mutants were constructed with substitutions of arginine to alanine in either the LLP1 (LLPM1) or the LLP2 (LLPM2) or in both domains LLP12 (LLPM3) to study the effects of certain conserved arginine residues for the structure and function of LLP12 domain. Both of main properties of LLP12, multimerization potential and cellular membrane-binding ability, were identified by comparison with these mutants. It proved that the mutations in LLP1 domain significantly decreased the multimerization potential of LLP12 protein (Figure 3), which may be due to the secondary structural alteration by two arginine substitutions in LLP1. Furthermore, all three mutants showed an impaired ability in binding to the outer membrane of 293T cells (Figure 4). However, none of these mutants notably affected their intermembrane targeting signal on LLP12. One possibility might be that certain membrane proteins were involved in this membrane-binding process, such as in the case of calmodulin (7,16–18). But this hypothesis was not further proved because of the limitation of our detection methods. Comparing our results with previous virus experiments by others, it seems that both important biochemical properties of LLP12 domain on HIV-1 gp41 could be involved in viral infectivity and the HIV-1 Env-mediated cell fusion. The different biochemical properties changes caused by respective mutations in LLP1 and LLP2 suggested their different roles in fusion reaction.

The Env gp160 precursor has the potential to form the oligomeric state after its synthesis and before its transport to the cell surface (19–21), and the multimerization of its LLP12 domain may be involved in this process. The three-dimensional structural differences between wide-type HIV-1 virions and cytoplasmic tail-truncated SIV virions also suggest a direct inter-Env interaction mediated by the cytoplasmic (22). In the present study, we purified the LLP12 segments with no tag add-on using bacterial expression system (Figure 2) and analyzed the multimeric potential of HIV gp41 LLP12 domain, which provided a more direct method to study the biochemical properties of this domain, comparing with the synthetic peptides or recombinant proteins (14,23,24). LLP12 and LLP2M (each with a MW of approximately 10 kDa) were eluted as complexes that were apparently larger than 150 kDa (Figure 3B). This finding indicated that they assembled into a higher-ordered form, such as possibly a tetramer of tetrameric forms. Similarly, we incubated the HeLa cells with LLP12 protein and examined the membrane-binding ability through anti-LLP12 sera, providing a more direct binding ability of LLP12 domain to cellular outermembrane.

The HIV-1 Env-mediated membrane fusion is a complicated process involving protein–protein interactions of different domains of gp41 and gp120. It has been reported that the Env mutant with a point mutation in the fusion domain dominantly interferes with fusion and infectivity (25). Like the N-terminal peptide, the biological properties of the usually long C-terminal cytoplasmic domain of gp41 influence the virus entry into host cells in various ways. Our present study showed the more direct evidence of some intrinsic features of LLP12 domain and provided the possible link between mutation caused defective in virus infectivity and changes in biological functions. It is likely that the LLP12 segment organize into highly ordered forms and such bind viral membranes to take part in the membrane fusion progress. Our study may provide insight into the structural and functional role of the conserved gp41 cytoplasmic LLP12 domain in membrane fusion and may help to unravel the complex process of Env-mediated virus–cell fusion.

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