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Original Article

Mutations of Conserved Glycine Residues within the Membrane-Spanning Domain of Human Immunodeficiency Virus Type 1 gp41 Can Inhibit Membrane Fusion and Incorporation of Env onto Virions

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SUMMARY: The membrane-spanning domain (MSD) of HIV-1 envelope protein (Env) has an additional glycine residue within a well-conserved putative transmembrane helix-helix interaction motif, GXXXG, and forms a $G^{690}G^{691}XXG^{694}$ sequence (G, glycine; X, any residues; the numbering indicates the position within the Env of an infectious molecular clone, HXB2). Different from vesicular stomatitis virus G (VSV-G), the glycine residues of the GXXXG motif of HIV-1 showed higher tolerance against mutations, and a simultaneous substitution of G690 and G694 with leucine residues only modestly decreased fusion activity and replication capacity of HIV-1. When G691 was further substituted with alanine, phenylalanine or leucine residue while G690 and G694 were substituted with leucine residues, the efficiency of membrane fusion decreased, with the decrease greatest occurring with the leucine substitution, a less severe decrease with phenylalanine, and the least severe decrease with alanine. Substitution with leucine residue also decreased the incorporation of Env onto virions, and the mutant showed the most delayed replication profile. Thus the presence of the extra glycine residue, G691, may increase the tolerance of the other two glycine residues against mutations than VSV-G. The fact that a more severe defect was observed for the leucine residue than the phenylalanine residue suggested that the function of Env depended on the steric nature rather than on the simple volume of the side chain of the amino acid residue at position 691. Based on this result, we propose a hypothetical model of the association among MSDs of gp41, in which G^{691} locates itself near the helix-helix interface.

INTRODUCTION

The envelope protein (Env) of human immunodeficiency virus type 1 (HIV-1) is a trimer of non-covalently associated heterodimers of gp120 and gp41. As with other retroviruses, gp120 (SU) and gp41(TM) play key roles in the determination of host range and membrane fusion, respectively.

For the three subdomains of HIV-1 gp41, the structure-function relationship of the ectodomain during membrane fusion has been elucidated at the molecular level (1,2). After gp120 binds to the receptor/coreceptor, the ectodomain undergoes a conformational change to form a six-helix bundle, a common structure observed for the class I fusion protein (3,4). Information on the structure-function relationships of the membrane-spanning domain (MSD) is rather limited. Although the amino acid sequence of the MSD of HIV-1's gp41 is highly conserved among different clades, the significance of the specific amino acid sequence within MSD has been underestimated, because some heterologous MSDs can substitute functionally for the native MSD of gp41 (5,6). However, truncation of HIV-1 MSD by a glycosylphosphatidylinositol anchor abolished the fusion activity (7). Furthermore, in viruses such as simian immunodeficiency virus and the influenza virus, mutations introduced into the MSD have been shown to impede the late stage of membrane fusion (8-11). These data suggest the importance of the MSD for the function of Env.

A glycine-containing helix-helix interaction motif, a GXXXG motif, has been found in MSDs of many membrane proteins such as glycophorin A (GpA) (12,13) and the hepatitis C virus envelope glycoproteins (14). In the case of HIV-1, it occurs as a $G^{690}G^{691}XXG^{694}$ sequence (G, glycine residue; X, any amino acid residue; the number indicates the position of each glycine residue in the Env of a molecular clone HXB2 [15]) within the MSD of gp41. The glycine residues within the GXXXG motif are critical for the proper fusogenicity of vesicular stomatitis virus G (VSV-G) (16). In a previous study we have shown that the point mutations of the individual glycine residue of the GXXXG motif of gp41 MSD were well tolerated (17). The molecular basis for this high tolerance of gp41 MSD against mutations was not identified.

Here we hypothesize that the GXXXG motif of gp41, like in other transmembrane helices, forms the helix-helix interface of gp41 MSDs. We reevaluated the role of glycine residues within the gp41 MSD by introducing a simultaneous substitution of several glycine residues. We confirmed the high tolerance of gp41 MSD against mutations through the finding that any combinatorial mutations of two glycine residues with leucine residues were well tolerated. By using one of the mutants, the $LG^{691}XXL$ mutant, we evaluated the role

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of the extra glycine residue by substituting it with several different amino acid residues, such as alanine, leucine, and phenylalanine residue. These substitutions negatively affected the function of Env, such as its fusogenicity or virion incorporation. We also found that there was a correlation between the steric characteristics of the side chain of the residue replacing G^{691} and the alteration in the function of Env. Based on these findings, we propose a potential association model of gp41 MSD.

MATERIALS AND METHODS

Construction of plasmids: The substitution mutants were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif., USA) using the subclone containing the 1.2-kb *Nhe*I-*Bam*HI fragment covering the *env* portion of HXB2RU3ΔN as described previously (17). The complementary oligonucleotide pairs used were as follow: 691F, ATGATAGTAG GATTCTTGGT AGGTTTA/ TAAA CCTACC AAGAATCCTA CTATCAT; 690/691-2L, GTACT GCTCT TGGTAGGTTT AAGAATAGTT TTTG/ CAAA AACTAT TCTTAAACCT ACCAAGAGCA GTAC; 690/694-2L, ATTCATAATG ATAGTACTGG GCTTGGTACT TTTAAG/ CTTAAAAGT ACCAAGCCCA GTACTATCAT TATGAAT; 691/694-2L, TTCATAATGA TAGTAGGACT CTTGGTACTT TTAAG/ CTTAAAAGTA CCAAGAGTCC TACTATCATT ATGAA. PCR was performed using PfuTurbo (Stratagene). The three glycine (G691) substitution mutants were created by site-directed mutagenesis using one subclone of the 2L mutants, 690/694-2L, as a PCR template and the following complementary oligonucleotide pairs: 690/694-2L + 691A, ATTCATAATG ATAGTACTGG CCTTGGTACT TTTAAG/ CTTAAAAGTA CCAAGGCCAG TACTATC ATT ATGAAT; 690/694-2L + 691L, CATAATGATA GTA CTGCTCT TGGTACTTTT AAGAAT/ ATTCTTAAAA GTACCAAGAG CAGTACTATC ATTATG; 690/694-2L + 691F, ATTCATAATG ATAGTACTGT TCTTGGTACT TTTAAG/ CTTAAAAGTA CCAAGAACAG TACTATC ATT ATGAAT). Following mutagenesis, the 1.2-kb *Nhe*I-*Bam*HI fragments were sequenced and cloned back into the pSP65HXB2RU3ΔN or pElucEnv (17) plasmid. The entire *Nhe*I-*Bam*HI portion, together with the junction, was verified by sequencing after the fragments were back.

Cells and antibodies: COS7 cells, 293 cells and 293CD4 cells (17) were grown in Dulbecco's modified essential medium (DMEM; Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah, USA) and penicillin-streptomycin (Gibco-BRL, Rockville, Md., USA). Jurkat cells were grown in RPMI 1640 (Sigma) supplemented with 10% FBS and penicillin-streptomycin. Cells were kept under 5% CO_2 in a humidified incubator. Anti-gp120 polyclonal antibody was obtained from Fitzgerald Industries International, Inc. (Concord, Mass., USA). The hybridoma 902 and Chessie 8 were obtained from Bruce Chesebro and George Lewis, respectively, through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (18-20). Serum from a patient infected with HIV-1 was kindly provided by T. H. Lee of Harvard School of Public Health, Boston, Mass., USA.

Protein analysis: The provirus DNA constructs were transfected into COS7 cells by electroporation or lipofection. In electroporation, COS7 cells (4 μg proviral DNA per 1×10^7 cells) were suspended in serum-free DMEM and electroporated at a 250-kV, 950-μF setting using Gene Pulsar II (Bio-Rad, Hercules, Calif., USA). In lipofection, COS7 cells (3×10^6 cells) were transfected with 7 μg proviral DNA by FuGene6 (Roche Molecular Biochemicals, Mannheim, Germany). At 72 h after transfection, transfected COS7 cells were collected by scraping and were centrifuged at 2,000 $\times$ *g* for 10 min (Allegra 6KR system; Beckman Coulter, Fullerton, Calif., USA). The cell pellets were dissolved in radioimmunoprecipitation assay (RIPA) lysis buffer (0.05 M Tris-Cl [pH 7.2] including 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) and the clear lysates were centrifuged at 314,00 $\times$ *g* for 45 min at 4°C (Himac CS 120fx system; Hitachi, Tokyo, Japan). The virus was sedimented from pre-cleared supernatants (centrifuged at 2,000 $\times$ *g* for 20 min; Allegra 6KR system, then filtered through 0.45-μm-pore-size filters; Millipore, Bedford, Mass., USA) by centrifuging at 113,000 $\times$ *g* for 1.5 h at 4°C on 3 ml of a 20% sucrose cushion (SW28 rotor; Beckman Coulter). Virus pellets were dissolved in RIPA lysis buffer. Both cell and virus lysates were run on a 7.5-15% gradient in a SDS-polyacrylamide electrophoresis gel (PAGE) system (DRC, Tokyo, Japan), and proteins were blotted onto Immobilon-P (Millipore) by passive transfer, as described previously (21). The immunoblotting procedure was as described previously (17). Enhanced chemiluminescence (Roche Molecular Biochemicals) and a LAS-3000 (Fuji Photo Film, Kanagawa, Japan) were used to detect the bands.

Infection study: For the infection study, the virus seed was prepared by transfecting 1 μg of the proviral DNA into 10^6 of the COS7 cells using FuGene6 (Roche Molecular Biochemicals). Jurkat cells were infected with each virus adjusted by the p24 amount (10 ng per 10^6 cells). The infection was monitored by measuring the amount of p24 in the culture supernatant at specific time points after infection (0, 8, 15, 22 and 25 days). A p24 ELISA was performed using a p24 RETRO-TEK ELISA kit (ZeptoMetrix, Buffalo, N.Y., USA).

Flow cytometry: The level of Env expressed on the cell surface was monitored by fluorescence-activated cell sorting analysis as described previously (17). Briefly, 48 h after the COS7 cells were transfected with each Env expression vector by FuGene6, the cells were stained with the 902 monoclonal antibody for 1 h at 4°C (10 μg/ml in phosphate-buffered saline [PBS] with 2% FBS), incubated with biotin-XX goat anti-mouse immunoglobulin G (Molecular Probes, Eugene, Oreg., USA) for 30 min at 4°C, treated with streptavidin Alexa Fluor 555 (Molecular Probes) for 30 min at 4°C, and finally fixed with 1% paraformaldehyde in PBS. Cells were suspended in PBS with 2% FBS and analyzed with Becton Dickinson FACSCalibur and CellQuest software (BD Biosciences Immunocytometry Systems, San Jose, Calif., USA). A double gate was defined by forward versus side scatter and by the amount of GFP (FL-1). A total of 10,000 events within this gate were collected for analysis. An Env KO that fails to express Env was used as a negative control, as described previously (17).

T7 RNA polymerase (RNApol) transfer assay: The efficiency of fusion pore formation was examined using T7 RNApol transfer assay as described previously (17). Briefly, COS7 cells were transfected with each Env expression vector together with the reporter plasmid pTM3hRL using FuGene6. The reporter plasmid contains the T7 promoter-driven renilla luciferase. At 48 h after transfection, the transfected COS7 cells were cocultured with the 293CD4 cells

that had been transfected with the T7 RNApol expression vector, pCMMP T7RNApoliresGFP (the ratio of cells was 1:1). At 12 h after the coculture, the cells were lysed. Firefly luciferase activities, derived from the Env expression vector, and renilla luciferase activities, activated by the T7 RNApol transferred from 293CD4 cells through the generated fusion pores, were determined using the Dual-Glo luciferase reporter assay system (Promega, Madison, Wis., USA).

RESULTS

Mutagenesis of glycine residues within the GGXXG sequence: In our previous study, mutations to an individual glycine residue within gp41 MSD were well tolerated, and high tolerance of gp41 MSD against mutations was expected (17). Therefore this time we simultaneously mutated several glycine residues within the MSD (Fig. 1). First, we substituted two glycine residues with leucine residues to create three forms of 2L mutants: 690/691-2L, 690/694-2L, and 691/694-2L. In the mutant 690/694-2L, the conserved glycine residues corresponding to the GXXXG motif were substituted. A similar mutation introduced into that of VSV-G resulted in functionally defective VSV-G (16). Next, to address the significance of the additional glycine residue at position 691, we substituted G691 with alanine, phenylalanine, or leucine residue while G690 and G694 were substituted with leucine residues. These substitutions formed the 2L + 691X mutants 690/694-2L + 691A, 690/694-2L + 691F, and 690/694-2L + 691L. Thus the sequence context of 2L + 691X mutants is LXxxL (the small x represents the original sequence of HXB2). As a control, the single substitution of the G691 with phenylalanine (691F) in which the other two glycine residues were left intact was generated (Fig. 1). The single substitution of the G691 to alanine (691A) or leucine residue (691L) was well tolerated and described in our previous study as mentioned above (17).

The replication profile of the MSD mutants: To examine replication capacity, Jurkat cells were infected with the mutant viruses after the p24 amount was adjusted for. Virus replication was monitored by measuring the amount of p24 released into the culture supernatants at 0, 8, 15, 22, and 25 days after infection. A representative result of the Jurkat cell experiment is shown in Figure 2. The single substitution mutant, 691F, was replicated with a slight delay compared with the wild type (WT). Other single substitution mutants, 691A or 691L, were replicated as efficiently as the WT (described in a previous study, data not shown) (17). The 2L mutants showed slightly delayed replication kinetics compared to those of the WT. The replication of the 2L + 691X mutants was less efficient than that of the WT. Substitution with leucine residue (690/694-2L + 691L) produced the slowest replication profile in repeated experiments. These data confirmed the high tolerance of the glycine residues within the GXXXG motif of gp41 MSD against mutation. Although the replication kinetics were generally slower in the H9 cells, a similar replication profile was observed (data not shown).

Analysis of the protein profiles of MSD mutants: The protein profiles of the mutants depicted in Figure 1 were examined by immunoblotting analysis using the serum from an individual infected with HIV-1 and anti-gp120 polyclonal antibody for both cell and virus lysates. Similar protein profiles were observed for all mutants and the WT in cell lysates (Fig. 3A). Almost equivalent amounts of gp160 and gp120 were observed for all constructs, and there was no obvious alteration in the processing. There was no difference found in the processing of Gag and Pol products. In the virus lysates, as in the cell lysates, no differences were found in the profiles of Gag and Pol products (Fig. 3B). However the amounts of gp120 found on 690/694-2L + 691L virions were 50 - 60% those of the WT (51.9% ± 14.4 %, $n = 3$, the amount of the incorporated Env was estimated by determining the ratio of the intensity of the Env to the p24 bands). This result is not due to the shedding of gp120, because the probing of the virus lysates with anti-gp41 monoclonal antibody detected a smaller amount of gp41 in 690/694-2L + 691L than in the WT (Fig. 3C). Therefore the alteration of the replication profiles observed for 690/694-2L + 691L (Fig. 2) may be accounted for by a defect in the incorporation of Env onto the virions. The reason other mutants manifested the slower replication is not evident from the protein profiles.

Fusion activity of mutants evaluated by the efficiency of fusion pore formation: Our previous study as well as others (11,17,22), have shown that mutations of the MSD sometimes negatively affect the fusogenicity of the Env. Therefore, to investigate the reason for the delayed replication observed in Figure 2, we evaluated the fusion efficiency of our mutants using the Env expression vector (Fig. 4A).

WT: yikLFIMIVGGLVGLRIVFAVLSIVnrv

691F: yikLFIMIVGFLVGLRIVFAVLSIVnrv

2L mutants

690/691-2L: yikLFIMIVLLLVGLRIVFAVLSIVnrv

690/694-2L: yikLFIMIVLGLVLLRIVFAVLSIVnrv

691/694-2L: yikLFIMIVGLLVLLRIVFAVLSIVnrv

690/694-2L+691 mutants

690/694-2L+691A: yikLFIMIVLALVLLRIVFAVLSIVnrv

690/694-2L+691L: yikLFIMIVLLLVLLRIVFAVLSIVnrv

690/694-2L+691F: yikLFIMIVLFLVLLRIVFAVLSIVnrv

Fig. 1. The mutants of gp41 MSD studied. The primary structures of the MSD mutants used in this study are shown using the one-letter abbreviation of amino acid residues. The position numbering is based on that used for HXB2 Env. The portion of the predicted MSD is shown in capital letters. WT corresponds to the wild type HXB2. Mutated residues are underlined.

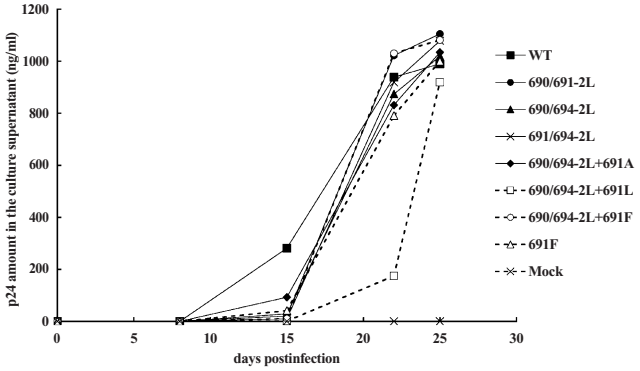

Fig. 2. The replication profile of gp41 MSD mutants in Jurkat cells. The replication of HIV-1 was monitored by measuring the p24 amount in the culture medium at 0, 8, 15, 22 and 25 days. The replication of 691A and 691L was similar to that of WT, as reported previously (17) and not shown here.

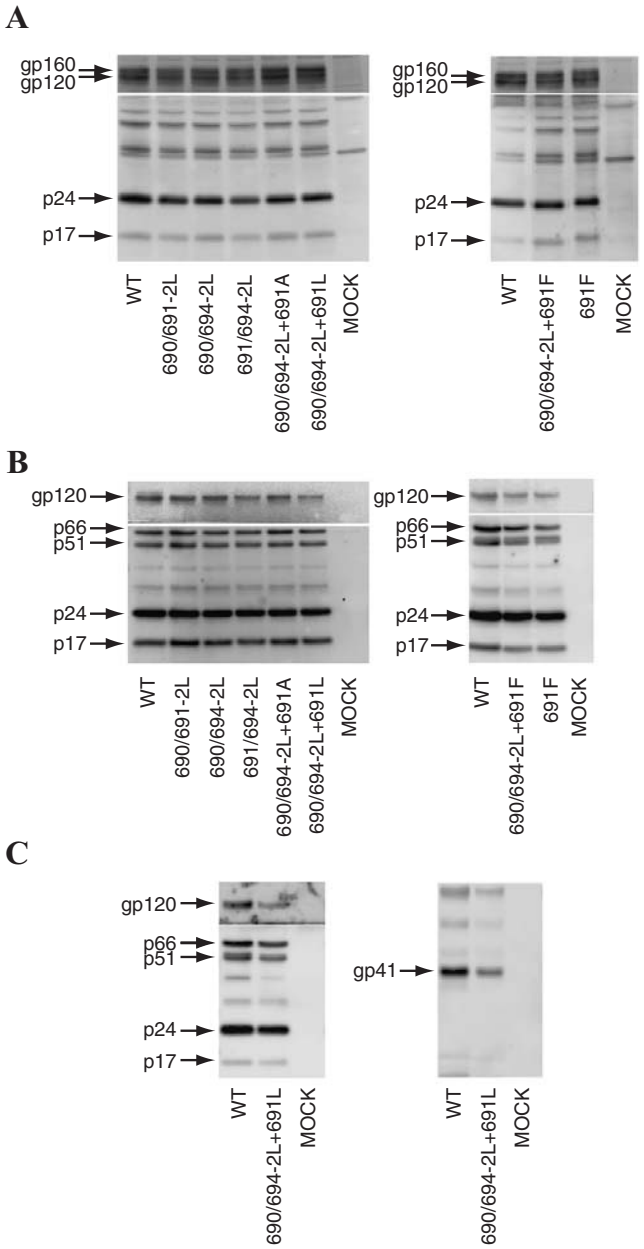

Fig. 3. Protein profiles of cell and virus lysates of the WT and mutants. The protein profiles of mutants were examined by immunoblotting. The cell (A) and virus (B, C) lysates prepared from transfected COS7 cells were used. The Env proteins (gp160, gp120 and gp41) were detected using anti-gp120 polyclonal antibody or anti-gp41 monoclonal antibody, respectively. Gag (p17 and p24) and Pol (p51 and p66) were detected using serum from an individual infected with HIV-1. The names of the mutants are shown.

First, we confirmed that the surface expression level of Env was similar by using the 902 monoclonal antibody in a flow cytometric analysis (Fig. 4B). This confirmation further supported the hypothesis that 690/694-2L + 691L has a defect in the incorporation of Env onto the virions rather than a defect in the intracellular transport of Env. We then analyzed the fusion activity of Env mutants using the T7 RNApol transfer assay. In this assay, the T7 RNApol that is transferred through the fusion pore between the Env- and receptor-expressing cells activates the T7 promoter-driven renilla luciferase. The renilla luciferase activity was normalized for transfection efficiency by the firefly luciferase activity derived from the Env expression vector. The representative data are shown in Figure 4C. Compared with WT, the 2L mutants showed a decrease in fusion efficiency of about 30%. There were no significant differences in fusogenicity among the different 2L mutants, a finding that was consistent with the replication profile shown in Figure 2. Therefore, the delayed replication observed for 2L mutants may be due to a defect in fusion. The delayed replication of 691F may also be due to a similar mechanism, although the observed decrease in the fusogenicity of 691F is not statistically significant (Fig. 4C).

The effect of the mutations of the conserved glycine residues within the GXXXG motif of gp41 MSD was less severe than in the case of VSV-G, in which a simultaneous substitution of two glycine residues corresponding to G690 and G694 resulted in almost complete elimination of fusion activity (16). The presence of an additional glycine residue within the GXXXG motif generates the $GG^{691}XXG$ sequence in gp41 MSD, which led us to evaluate the effect of substituting other amino acid residues for the glycine residue at position 691 in the 690/694-2L context for membrane fusion. Substituting glycine residue with alanine residue did not reduce the fusion efficiency further (for example, compare 690/694-2L with 690/694-2L + 691A in Fig. 4C). However, changing the glycine residue to phenylalanine reduced the fusion efficiency significantly (690/694-2L versus 690/694-2L + 691F in Fig. 4C). The introduction of leucine residue in place of the glycine residue had the most severely negative effect. Thus the presence of a glycine residue at position 691 seems to produce an apparently higher fusion efficiency for gp41 even when the two other glycine residues constituting the GXXXG motif were replaced with leucine residue. Furthermore, because substitution with leucine resulted in less fusogenic gp41 than did substitution with phenylalanine residue, it seemed that the steric nature of the side chain at position 691, not simple bulkiness, affected the outcome.

DISCUSSION

The importance of the GXXXG motif in helix-helix association has been well established through the studies of GpA. The glycine residues within the GXXXG motif play a critical role in helix-helix interaction (13,23). Here we showed that the mutations introduced in the conserved glycine residues within the GXXXG motif of gp41 MSD were well tolerated. This finding is quite different from that of the previous study of VSV-G MSD, where a similar mutation corresponding to the 690/694-2L mutant almost abolished the fusion activity (16). Furthermore, we also observed that the substitution of any two glycine residues within the GGXXG sequence of gp41 MSD only modestly decreased the fusion activity (Fig. 4C). The expression, processing, and transport of Env proteins to the cell surface were preserved (Fig. 3A and Fig. 4B). Consistent with the modest decrease in fusion activity, the replication profiles of these mutant viruses were only slightly retarded when compared to that of the WT virus (Fig. 2). This result confirmed the high tolerance of gp41 MSD that allows the substitution of any two glycine residues within the GGXXG sequence. The results for the 690/694-2L mutant may suggest that the GXXXG motif of gp41 MSD does not play a role as a helix-helix interaction motif or that there is another mechanism that cancels out the introduced mutations.

The presence of the additional glycine residue within the GXXXG motif is a well-conserved feature of gp41's MSD (15), and this feature is absent from VSV-G. The three

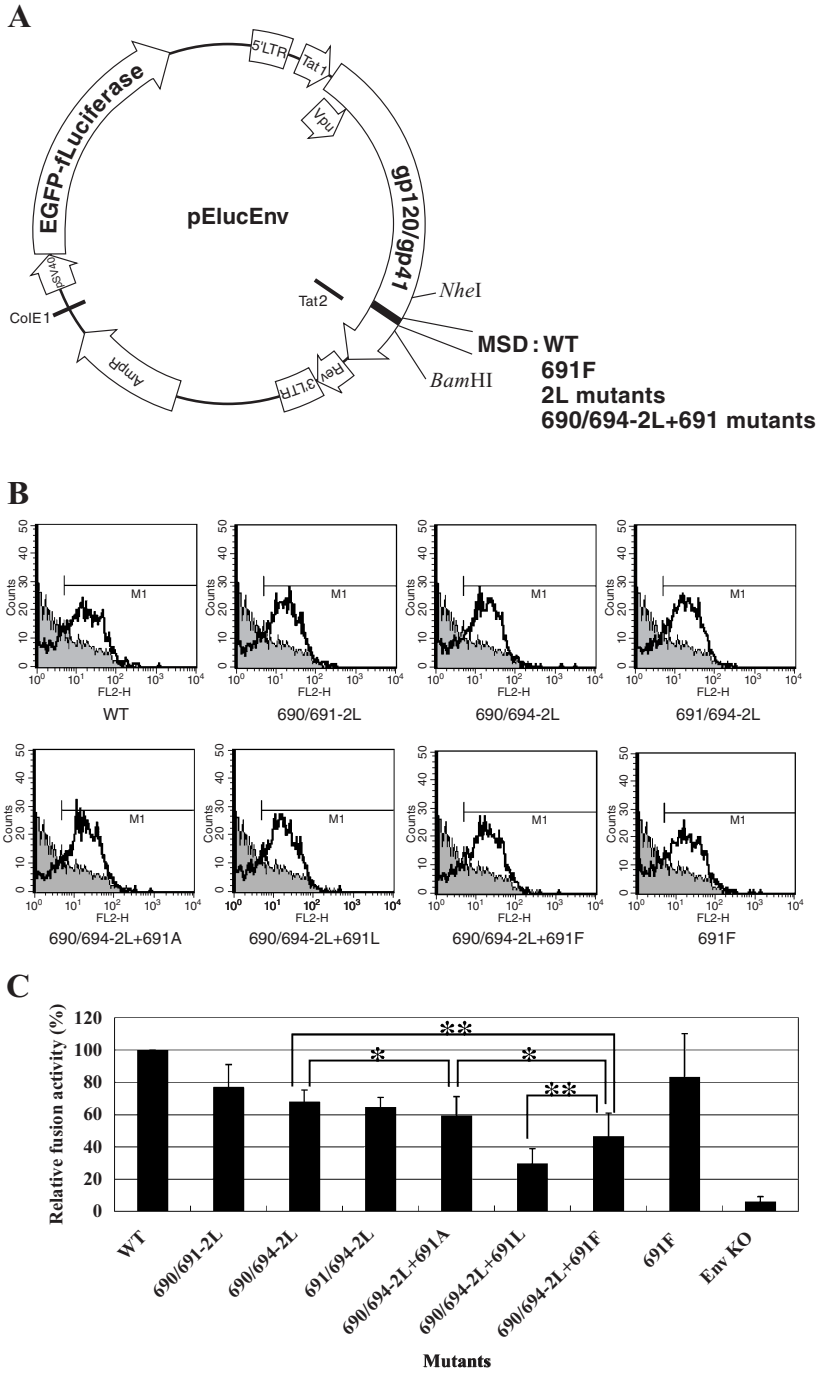

Fig. 4. Evaluation of the cell surface expression and measurement of the fusion activity of mutant Env. (A) The map of the Env expression vector, pElucEnv. pElucEnv supports the expression of HIV-1 *env* (gp120/gp41) and the gene of the EGFP-firefly luciferase (EGFP-fLuciferase) hybrid protein, respectively, from two separate promoters. The *Nhe*I and *Bam*HI sites used for cloning are indicated. The LTR, *tat*, *vpu*, and *rev* of HIV-1 are shown. AmpR, β-lactamase gene; SV40, SV40 late promoter; ColE1, ColE1 replication origin. (B) Cell-surface expression of Env. FACS analysis of Env expressed on the surface of COS7 cells transfected with each pElucEnv construct was accomplished as described in the Materials and Methods section. The signal for each Env is shown as a gray line. The filled area depicts the signal obtained for the control vector, Env KO. (C) Fusion activity of the mutants evaluated by T7 RNApol transfer assay. The cell-cell fusion assay between the Env expressing cells (containing T7 promoter-driven plasmid) and CD4+ cells (bearing the T7 RNApol expression plasmid) was used to evaluate the fusion efficiency of mutant Envs. A representative result of four independent experiments is shown (*statistically not significant, **statistically significant difference: $P < 0.05$ by Student's *t* test). The results shown are means ± s.d. ($n = 4$).

glycine residues will cluster within the gp41 MSD helix (Fig. 5A, prepared by the program Insight II; Accelrys, San Diego, Calif., USA). Having a hydrogen atom as its side chain, the clustering of three glycine residues may give the region more flexibility to accommodate mutations. To test whether the high tolerance of gp41MSD against mutations is attributable to the presence of the extra glycine residue within the gp41 GXXXG motif, we further mutated the extra glycine residue (G691) while the other two glycine residues were mutated to leucine residues under the LG^{691}XXL context. This also allows us to obtain information on a potential helix-helix interface among gp41 MSDs. We would expect the mutation

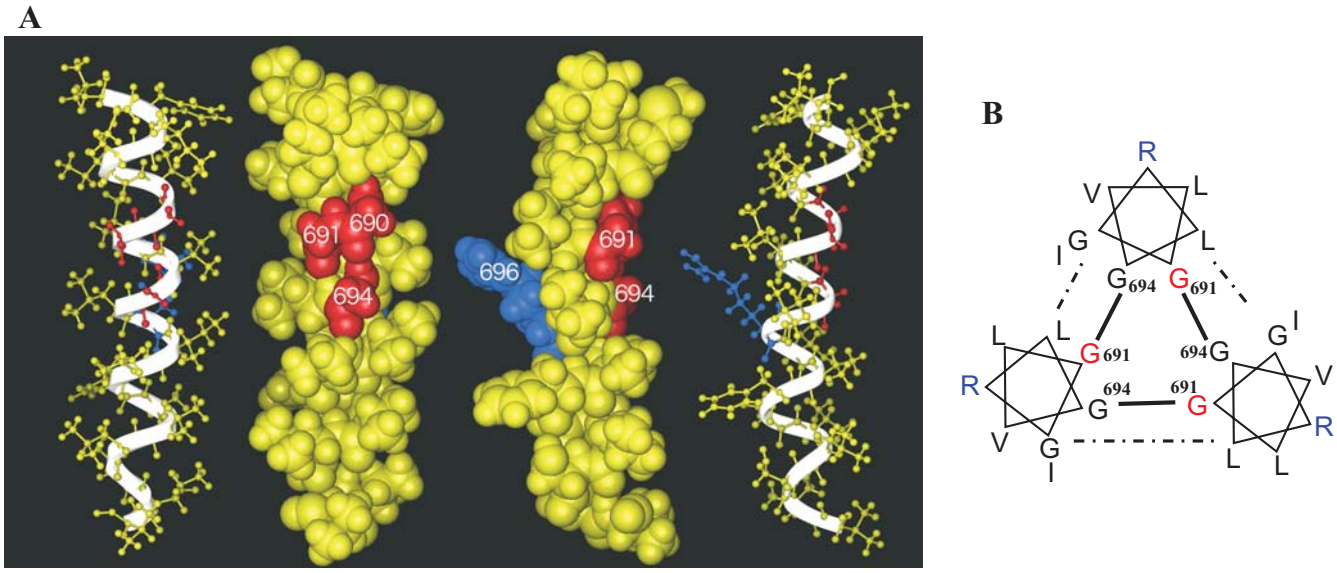

Fig. 5. A structural model of MSD and a hypothetical arrangement of the MSD helices. (A) The MSD region (position 684-705 of HXB2 Env, the amino acid sequence: LFIMIVGGLVGLRIVFAVLSIV) of the WT HXB2 was modeled in an a-helix conformation. Molecular dynamics simulations were carried out in the lipid-like environment, using the Generalized Born method with a relative permittivity of 4.0 (25, 26). The most representative structure seen in the simulation was extracted by a principal component analysis and is shown graphically. Three glycine residues (shown in red) colocalize each other and generate a hollow surface on one aspect of the helix. Two different views (ball-and-stick and CPK) of the same helix are shown. (B) A hypothetical arrangement of the MSD helices. The glycine residue at positions 691 and arginine residue at position 696 are shown in red and blue, respectively. The potential interactions between amino acid residues are shown by the bold and dotted lines, respectively.

to have no significant effect if G691 was facing outward to the lipid environment and was not involved in the helix-helix interaction. We took a genetic approach and we created the mutants 690/694-2L + 691A, 690/694-2L + 691F, and 690/694-2L + 691L. None of these mutations changed the processing or surface expression of Env (Figs. 3A and 4B). This suggests that these mutations did not induce drastic conformational changes that could be detected by a quality control mechanism of endoplasmic reticulum. Indeed, our molecular dynamics analyses of these mutants failed to detect severe deformation of the structure of the individual helix (data not shown).

When the fusion activity of the 690/694-2L + 691X mutants was evaluated, there was no statistically significant difference between glycine and alanine residue (Fig. 4C). This finding is consistent with the observation that alanine residue can functionally substitute glycine residue in transmembrane helices (23). The substitution of glycine residue with a bulky residue, namely phenylalanine residue (690/694-2L + 691F), resulted in a significant reduction in fusion activity. The fusion efficiency of 690/694-2L + 691F was about 50% that of WT. Substitution with leucine residue (690/694-2L + 691L) had an even greater negative effect on fusogenicity, and the fusion efficiency of 690/694-2L + 691L was about 30% that of WT (Fig. 4C). The replication efficiency somewhat reflected these changes in fusion activity, although we could not attribute the defect of 690/694-2L + 691L to the defect in fusion alone because 690/694-2L + 691L had an additional defect in Env incorporation (Figs. 3B and C). These results showed that the presence of glycine residue at position 691 seemed to reduce the negative effects of the leucine residue substitution of glycine residue at positions 690 and 694.

It is noteworthy that we have observed the phenotypic changes of gp41 according to the nature of the substituted amino acid residue at the position 691 (Figs. 2-4). Interestingly, the negative effect of mutations was not dependent on the mere volume of the side chain (F > L), but rather on the steric nature of the side chain. The introduction of another branched amino acid residue, isoleucine residue, at position 691 also resulted in decreased fusion activity (data not shown). This may suggest that the mutation may affect the interhelix association of MSDs, and the changes in association among MSDs may affect the function of gp41. Indeed our preliminary analysis of the measurement of helix-helix interaction by means of TOXCAT (12) analysis indicated that 690/694-2L + 691L had a slightly stronger association than WT (E. Matthews and D. M. Engelman, unpublished data). Based on these data, we present our hypothetical model of the association of the three MSDs in which the GXXXG motif is facing inward as shown in Figure 5B. Under this configuration, the G691 locates itself near the helical interface. Although this model is consistent with our observation, it places the highly conserved arginine residue downstream of the GXXXG motif toward the lipid environment. This may not be a thermodynamically favorable arrangement despite the recent finding that suggested that arginine residue can be accommodated into a lipid bilayer more easily than previously expected (24). Therefore we cannot rule out alternative arrangements of the gp41 MSDs at present. The observed high tolerance of glycines constituting this hypothetical interface may also suggest that the potential interaction among gp41 MSDs may be a rather weak one. To prove our model, the physical structure of the gp41 MSDs in lipid environments must be determined.

Here we have shown that the efficiency of the Env-mediated fusion pore formation and the incorporation of Env onto virions were affected by alterations within the MSD of gp41. This confirms that the specific primary structure of MSD is important for its proper function. Our findings also suggest that a subtle change in the structure within MSD can affect the conformations of other subdomains of gp41. Conversely, they may suggest that the conformational changes

in other subdomains may affect the structure of the MSD. This may have implications for the mechanism of disassembly and for that of uncoating events, and may also suggest that the MSD of gp41 may become another target for therapeutic intervention against HIV-1 infection.

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