Identification of the HIV-1 gp41 Core-binding Motif in the Scaffolding Domain of Caveolin-1*

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Jing-He Huang,‡ Lu Li,§ Hong Lu, Xi Chen,‡ Shibo Jiang,§, and Ying-Hua Chen†‡

From the ‡Laboratory of Immunology, Department of Biology, Tsinghua University, Protein Science Laboratory of the Ministry of Education, Beijing 100084, P.R. China and the §Laboratory of Viral Immunology, Lindsley F. Kimball Research Institute, New York Blood Center, New York, New York 10021

The human immunodeficiency virus, type 1 (HIV-1) gp41 core plays an important role in fusion between viral and target cell membranes. A single chain polypeptide, N36(L8)C34, which forms a six-helix bundle in physiological solution, can be used as a model of gp41 core. Here we identified from a 12-mer phage peptide library a positive phage clone displaying a peptide sequence with high binding activity to the HIV-1 gp41 core. The peptide sequence contains a putative gp41-binding motif, ΨXXXΨΨΨX (X is any amino acid residue, and Ψ is any one of the aromatic amino acid residues Trp, Phe, or Tyr). This motif also exists in the scaffolding domain of caveolin-1 (Cav-1), a known gp41-binding protein. Cav-1-(61–101) and Cav-1-(82–101), two recombinant fusion proteins containing the Cav-1 scaffolding domain, bound significantly to the gp41 expressed in mammalian cells and interacted with the polypeptide N36(L8)C34. These results suggest that the scaffolding domain of Cav-1 may bind to the gp41 core via the motif. This interaction may be essential for formation of fusion pore or endocytosis of HIV-1 and affect the pathogenesis of HIV-1 infection. Further characterization of the gp41 core-binding motifs may shed light on the alternative mechanism by which HIV-1 enters into the target cell.

Entry of human immunodeficiency virus type 1 (HIV-1)§ into the target cell is triggered by interaction of the viral envelope glycoprotein (Env) surface subunit gp120 with the primary receptor CD4 (1) and a chemokine co-receptor (CXCR4 or CCR5) (2), resulting in a series of conformational changes of the Env transmembrane subunit gp41, i.e. insertion of the gp41 N-terminal fusion peptide into the target cell membrane, and association between the N- and C-terminal heptad repeat (NHR and CHR, respectively) regions to form a six-helix bundle (6-HB; also known as trimer-of-hetero-dimers or trimer-of-hairpins) consisting of three NHR helices as the central trimeric coiled-coil domain and three CHR helices as the outer coiled-coil layer (3–5).

Peptide derived from the gp41 NHR region (designated N-peptides), e.g. N36, and CHR region (named C-peptides), such as C34, when mixed in physiological solution, can form a stable 6-HB, representing the gp41 core structure (6, 7). Similarly, the single chain polypeptide N36(L6)C34 in which the C terminus of N36 is connected to the N terminus of C34 by a 6-amino acid linker (SGGGRGG) could also form the trimer-of-hairpins structure (8). Therefore, this polypeptide has been widely used as a model of the gp41 core in the study of the HIV fusogenicity (9–11).

Formation of the 6-HB by viral NHR and CHR is believed to bring the viral and target cell membranes into proximity for fusion (3–5, 12). However, Markosyan et al. (13) conceive that the essential role of the gp41 core is to stabilize the fusion pore against collapse and ensure its growth, because formation of some 6-HBs occurs after a fusion pore has formed. It is also speculated that gp41 core may participate in the formation of the fusion pore in the target cell membrane (14). Lu and colleagues (15) reported that the gp41 6-HB structure could interact with the lipids in cellular membranes. But the fate of the gp41 core and whether this core structure interacts with any cellular protein during the membrane fusion process have not been determined.

We are interested in searching for the gp41 core-binding motifs in cellular proteins. By screening a 12-mer phage display peptide library with N36(L8)C34 (L8 = GGGKLGGG), we identified an amino acid (aa) sequence, WHWTSYLY (9021), which contains a putative gp41-binding motif, ΨXXXΨΨΨX. Caveolin-1 (Cav-1), a known gp41-binding protein, also contains the same motif in its scaffolding domain. Two recombinant proteins, including the cytoplasmic membrane proximal region (aa 61–101) and the scaffolding domain (aa 82–101) of Cav-1, could bind to gp41 in the pull-down and co-immunoprecipitation assays and interact with the 6-HB of gp41 modeled by N36(L8)C34. These results suggest that the ΨXXXΨΨΨX motif presented in the Cav-1-(82–101) scaffolding domain may be responsible for the interaction between...
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Cav-1 and the gp41 core, which may be involved in the process of fusion between the HIV-1 envelope and the target cell membrane, or endocytosis of HIV-1 particles. Binding of gp41 core to Cav-1 may affect pathogenesis of HIV-1 infection.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transient Transfection**—3T3 cells stably transduced with MLV MX-CD4 and MX-CXCR4 vectors (3T3.T4.CXCR4) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen). CHO cells stably transduced with the HIV-1Env-expressing vector pEE14 (CHO-WT) or control pEE14 vector (CHO-EE) were cultured in glutamine-deficient minimal essential medium containing 400 μM methionine sulfoximine (Sigma). 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with the antibiotics and 10% fetal bovine serum. Transfection was performed using Vigo-fect reagent (Vigorous Biotechnology, Inc., Beijing, China) according to the manufacturer’s protocol. Cells were plated in a 6-well tissue culture plate 24 h prior to transfection. Cells at 50–70% confluency were transfected with 5 μg of plasmid DNA. Cells were collected 48 h after transfection.

**Phage Display Peptide Screening**—Peroxidase-conjugated anti-M13 phage antibody was purchased from Amersham Biosciences. Ph.D.-12 phage display peptide libraries were obtained from New England Biolabs (Beverly, MA). Biopanning was performed following the manufacturer’s standard procedure. The polypeptide N36(L8)C34 expressed and purified as previously described (17) was used to coat microtiter plates at 10 μg/ml. After three rounds of selection, the bound phages were eluted with glycine-HCl buffer (pH 2.2). The selected clones were added to Escherichia coli (strain ER2738) culture (1:100) for infection at 37 °C for 5 h with shaking. The culture supernatants containing phage particles were harvested as phage stocks and stored at −20 °C until use.

**Peptide Synthesis**—The peptides JCH-1 (see its sequence in Table 1) was synthesized by a standard solid-phase Fmoc (N-(9-fluorenylethoxycarbonyl) method as previously described (8). Its N and C termini were acetylated and amidated, respectively. This peptide was purified to homogeneity by high-performance liquid chromatography. The identity of the purified peptide was confirmed by laser desorption mass spectrometry (PerSeptive Biosystems).

**Enzyme-linked Immunosorbent Assay**—To determine the activity of the phage clones binding to the polypeptide N36(L8)C34, wells of microplates were coated with 50 μl of N36(L8)C34 (0.5 μg/ml) in 0.1 M NaHCO3 buffer (pH 8.6) overnight. The coated wells were blocked with Tris buffer solution (TBS, pH 7.5) containing 0.25% gelatin (additional uncoated control wells were blocked with TBS containing 0.25% gelatin to exclude the selected clones that bind gelatin-coated plastic). After three washes with TBS containing 0.1% Tween-20 (TBS-T), phages diluted in TBS at a series of 5-fold (starting from 1011 particles/ml) were added to the wells, followed by incubation at room temperature for 1.5 h with agitation. After extensive washes, the amount of bound phage was detected by addition of peroxidase-conjugated anti-M13 phage antibody and substrate, o-phenylenediamine, sequentially. The absorbance at 450 nm (A450) was recorded.

To determine the binding activity of the peptide JCH-1 to N36(L8)C34, wells of microplates were coated with JCH-1 (5 μM). After blocking and washes, N36(L8)C34 at 4-fold serial dilutions (starting from 5 μM) was added. After incubation at room temperature for 1.5 h and extensive washes, the bound peptides were detected by addition of 50 μl of rabbit anti-N36/C34 antibody and peroxidase-conjugated anti-rabbit IgG and substrate o-phenylenediamine, sequentially. The A450 was measured.

To test whether JCH-1 could compete with the Cav-1 (61–101) or Cav-1 (82–101) to bind the 6-HB, wells of microplates were coated with 50 μl of Cav-1 (61–101) or Cav-1 (82–101) (10 μg/ml). After blocking and washes, 50 μl of the JCH-1 or an unrelated control peptide-NM2 (KSSLGEVETTIRKSLGEGEVTTR, a peptide derived from the integral membrane protein M2 of influenza A virus), at 4-fold serial dilutions in PBS was added, followed by addition of 25 μl of N36(L8)C34 (10 μg/ml) and incubated at room temperature for 1 h with agitation. After extensive washes, the amount of bound N36(L8)C34 was detected by addition of rabbit anti-N36/C34 polyclonal antibodies and peroxidase-conjugated anti-rabbit IgG and substrate o-phenylenediamine, sequentially. The absorbance at 450 nm (A450) was recorded.

**DNA Sequencing**—The positive phage clones were precipitated with polyethylene glycol. The DNA samples were prepared by phenol extraction, and the DNA sequences were determined using primer provided in the Ph.D. kit (Biolabs).

**Surface Plasmon Resonance Assay**—The kinetics of the binding affinity of JCH-1 to N36(L8)C34 were determined by SPR using BLAcore 2000 system (Amersham Biosciences) at 25 °C. N36(L8)C34 (10 μg/ml) was immobilized onto the CM5 sensor chip according to amine-coupling protocol, and the unreacted sites were blocked with 1 M Tris-HCl (pH 8.5). The association reaction was initiated by injecting JCH-1 at a flow rate of 5 μl/min. The dissociation reaction was done by washing with PBS. At the end of each cycle, the sensor chip surface was regenerated with 0.1 M glycine-HCl (pH 2.5) for 30 s. Binding of
GST-Cav-1-(61–101), GST-Cav-1-(82–101), and GST-Cav-1-(61–88), respectively, to N36(L8)C34 were also measured with the same procedure as described above.

Expression and Purification of GST-Cav-1-(61–101), GST-Cav-1-(61–88), and GST-Cav-1-(82–101) Fusion Proteins—For expression of GST-Cav-1-(61–101) and GST-Cav-1-(82–101) fusion proteins, the gene fragments encoding the Cav-1-(61–101) and Cav-1-(82–101) were amplified with PCR from the genome of U937 cell with the primers: Cav-1-(61–101) F: 5’-CGGAATTCGATGACGTGGTCAAGATTG-3’; Cav-1-(82–101) F: 5’-CGGACCGGATTTGGAGGCC-3’ and R: 5’-CCGCTCGAGTCAGCGGTAAAACCAGTATTTCG-3’. The fragments were cloned into the expression plasmid PGEM-6P via its EcoRI and XhoI restriction sites, and the proteins were overproduced in E. coli strain Rosetta. The cells were lysed in PBS (pH 7.2) by sonication. After centrifugation, the supernatants containing the fusion protein were collected. The GST-Cav-1-(61–101) and GST-Cav-1-(82–101) fusion proteins were then purified by glutathione-Sepharose 4B affinity columns and were analyzed by SDS-PAGE. GST-Cav-1-(61–101) and GST-Cav-1-(82–101) was constructed and expressed as a control with the same procedure as described above.

Pull-down Assay—The in vitro pull-down assay was carried out as described previously (16). Equal amounts of GST-Cav-1-(61–88), GST-Cav-1-(61–101), GST-Cav-1-(82–101), or GST-conjugated glutathione-Sepharose beads were incubated with 5 μg of N36(L8)C34 on ice for 2 h. The beads were washed five times with PBS containing 0.05% Tween-20 (PBS-T). The bound 6-HB was detected by Western blot with the rabbit polyclonal anti-GST antibody.

Immunoprecipitation Assay—The pellet of the 293T cells containing the EGFP-tagged Cav-1-(82–101) and human IgG Fc-tagged rsgp41 was solubilized in 0.2 ml of ice-cold lysis buffer at 4 °C for 30 min. The supernatants were collected after centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants containing gp41 ectodomain were collected and incubated with GST and GST-Cav-1-(61–101)-conjugated glutathione-Sepharose beads, respectively, on ice for 2 h. The beads were washed with PBS-T five times. The bound gp41 was eluted by heating with SDS-PAGE sample buffer and detected by Western blot with the polyclonal anti-N36/C34 antibody. Similar procedures were used for pulling down the complex of GST-Cav-1-(61–101) with human IgG Fc-tagged rsgp41 expressed in the transfected 293T cells. The bound GST-Cav-1-(61–101) was detected by the rabbit polyclonal anti-GST antibody.

Immunoprecipitation Assay—The pellet of the 293T cells containing the EGFP-tagged Cav-1-(82–101) and human IgG Fc-tagged rsgp41 was solubilized in 0.2 ml of ice-cold lysis buffer at 4 °C for 30 min. The supernatants were collected after centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants containing gp41 ectodomain were collected and incubated with GST and GST-Cav-1-(61–101)-conjugated glutathione-Sepharose beads, respectively, on ice for 2 h. The beads were washed with PBS-T five times. The bound gp41 was eluted by heating with SDS-PAGE sample buffer and detected by Western blot with the polyclonal anti-N36/C34 antibody. Similar procedures were used for pulling down the complex of GST-Cav-1-(61–101) with human IgG Fc-tagged rsgp41 expressed in the transfected 293T cells. The bound GST-Cav-1-(61–101) was detected by the rabbit polyclonal anti-GST antibody.

### TABLE 1

**Amino acid sequences of the peptides containing the potential gp41 core-binding motif**

| Peptide sequence of the phage clone gcb-1 | JCH-1 peptide sequence |
|------------------------------------------|------------------------|
| | Cav-1-(61–101) |
| | Cav-1-(82–101) |

Note: the amino acid sequences of the peptides containing the potential gp41 core-binding motif are shown. Motif

DGIWKASFTTVTKYR

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**FIGURE 2. Binding of the synthetic peptide JCH-1 to the 6-HB.**

A. binding of JCH-1 peptide to N36(L8)C34 measured by sandwich ELISA. B, binding affinity of JCH-1 peptide to N36(L8)C34 measured by SPR. N36(L8)C34 was immobilized onto the sensor chip CM5. Binding of JCH-1 (starting from 233 μM) to N36(L8)C34 was measured by SPR using the Biacore 2000 system. An irrelevant peptide at 233 μM was included as a negative control.
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HIV-1 Env-mediated Syncytium Formation Assay—Target (3T3.T4.CXCR4) cells (5 × 10^4) resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum were plated in 96-well plates and incubated overnight at 37 °C, followed by washes with glutamine-deficient minimal essential medium. Then, 2 × 10^4 effector (CHO-WT) cells pre-stimulated with 7 mM sodium butyrate for ~20 h were added in the absence or presence of an inhibitor at granted concentration. After co-cultured for 24 h at 37 °C, the syncytia, defined as giant cells with diameters more than four times bigger than those of single cells, were counted under a microscope. The percentage of inhibition of syncytium formation was calculated using the formula: % inhibition = (1 - (number of syncytia in a well containing an inhibitor)/number of syncytia in a well containing no inhibitor) × 100. To determine the biological function of JCH-1, JCH-1 peptide (starting from 400 µg/ml) was preincubated with CHO-WT cells at 37 °C for 60 min, followed by addition of the mixture to the target cells. Other steps were the same as above. The concentration for 50% inhibition (IC50) was calculated using the CalcuSyn software (34).

RESULTS

Identification of a gp41 Core-binding Sequence—A single chain polypeptide N36(L8)C34, in which the C terminus of N36 is connected to the N terminus of C34 by the hydrophilic linker, GGGLGLGG (L8), was used as a model of the gp41 core for screening of the 12-mer phage peptide library. As confirmed by a sandwich ELISA using the gp41 core-specific monoclonal antibody NC-1 (8), this polypeptide formed a stable 6-HB (17). After three rounds of biopanning, five positive clones were observed. One of the positive clones with the highest gp41 core binding activity, designated gcb-1, was selected for further

**TABLE 3**
Proteins containing the potential gp41 core-binding motifs

| Protein | The 1st residue of the motif | Potential gp41 core-binding motifs: |
|---------|-----------------------------|-----------------------------------|
| Caveolin | 89 | ΦXXXΦΦΨΦ |
| IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channel | 2452 | ΦSTVYΦΦ |
| Heparan sulfate N-deacetylase | 32 | ΦISAXΦΦ |
| Heparan sulfate N-sulfotransferase | 52 | ΦISAXΦΦ |
| γ-Glutamylcarboxylase | 390 | ΦTNGLΦΦ |
| Ephrin receptor EphA1 | 810 | ΦWSFGVWL |
| EPHA2 protein | 78 | ΦWSFGVWL |
| Insulin receptor variant | 671 | ΦWSFGVWL |
| Insulin receptor-related receptor | 1147 | ΦWSFGVWL |
| Insulin-like growth factor 1 receptor, IGF-IR | 1196 | ΦWSFGVWL |
| Neurotrophic tyrosine kinase receptor type 1 | 710 | ΦWSFGVWL |
| Hepatocyte growth factor receptor precursor | 1 | ΦWSFGVWL |
| Receptor tyrosine kinase-like orphan receptor 1 | 676 | ΦWSFGVWL |
| Neurotrophic tyrosine kinase, receptor, type 1 isoform 1 | 705 | ΦWSFGVWL |
| Mixed lineage kinase ZAK | 190 | ΦWSFGVWL |
| RIP protein kinase | 212 | ΦYSFAVVL |
| RIP3 γ | 205 | ΦYSFILL |
| Receptor (TNFRSF)-interacting serine-threonine kinase 1 | 212 | ΦYSFAVVL |
| Receptor-interacting serine-threonine kinase 3 | 205 | ΦYSFILL |
| Receptor tyrosine kinase | 1 | ΦWSFGVWL |
| MLTK-α (MAP kinase kinase kinase) | 190 | ΦYSFAVVL |
| Mitogen-activated protein kinase kinase kinase 12 | 294 | ΦWSFGVWL |
| MAPK12 protein | 327 | ΦWSFGVWL |
| MAP3K13 protein | 27 | ΦWSFGVWL |
| MET proto-oncogene protein | 1285 | ΦWSFGVWL |
| Sodium-dependent high affinity dicarboxylate transporter | 254 | ΦWSFGVWL |
| SLC13A3 (sodium-dependent dicarboxylate transporter) | 207 | ΦWSFGVWL |
| Solute carrier family 13 member 3 isoform a | 294 | ΦWSFGVWL |
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The sequence WHWTSYLGYGWSS presented in the phage clone gcb-1 and the synthetic peptide JCH-1 can specifically bind to the HIV-1 gp41 core domain and may interfere with the gp41 core-mediated membrane fusion process.

Interaction between the gp41 Core and the Cytosolic Membrane-proximal Region of Cav-1—Analyzing the sequence of WHWTSYLGYGWSS, we noticed that it contained two motifs, ΦXXXΦXXΦ and ΦXXΦXXXΦ. By searching the protein data base, we found that five proteins (Cav-1, IP3-sensitive Ca2+ channel, heparan sulfate N-deacytelase, heparan sulfate N-sulfotransferase, and γ-glutamyl-carboxylase) contained the motif ΦXXXΦXXΦ and about two dozen proteins consisted of ΦXXΦXXXΦ motif (Table 3). We then searched the reports in Medline against each of these proteins and found that only Cav-1 was reported to bind with the HIV-1 gp41 (18). We thus speculated that the Cav-1 might also bind to the gp41 core modeled by the N36(L8)C34. To investigate this possibility, the Cav-1 cytosolic membrane-proximal region, which participates in homo-oligomerization (19), was constructed as a GST fusion protein, GST-Cav-1-(61–101). This fusion protein was expressed in E. coli and purified using glutathione-Sepharose 4B affinity column. The purified GST-Cav-1-(61–101) migrated through the SDS-PAGE at an apparent size of 31 kDa (Fig. 4A).

To determine the interaction between Cav-1-(61–101) and gp41, CHO-WT cells expressing the HIV-1 Env gp120/gp41 and the control CHO-EE cells, which express no Env, respectively, were incubated with 5 μg/ml of soluble CD4 at 37 °C for 30 min to induce shedding of gp120 and an increased exposure of gp41 (20). The lysates of CHO-WT cells were incubated with GST or GST-Cav-1-(61–101) conjugated to glutathione beads. The pull-down proteins were separated by SDS-PAGE and probed by polyclonal antibodies against the mixture of the peptides N36 and C34 derived from HIV-1 gp41 NHR and CHR regions, respectively. As shown in Fig. 4B, GST beads pulled down no protein (lane 1), whereas GST-Cav-1-(61–101)-beads precipitated gp41 from the CHO-WT cell lysates (lane 2). No protein from the control CHO-EE cell lysates was captured by GST (lane 3) and GST-Cav-1-(61–101) (lane 4). This result suggests that the

study. As shown in Fig. 1, gcb-1 bound significantly to N36(L8)C34 in a dose-dependent manner, whereas the control M13 bacteriophage showed no binding with N36(L8)C34. By analyzing its sequence, we found that this clone exhibited the following peptide sequence: WHWTSYLGYGWSS. To determine whether this sequence is responsible for binding of the phage clone gcb-1 to N36(L8)C34, we synthesized a peptide, designated JCH-1, containing this sequence (Table 1) and tested its activity of binding with the 6-HB modeled by N36(L8)C34 in the sandwich ELISA with the gp41 core-specific monoclonal antibody NC-1. Like the phage clone gcb-1, the synthetic peptide JCH-1 also bound to N36(L8)C34 in a dose-dependent fashion (Fig. 2A). We further measured its binding affinity using a 293T cells + Protein G beads. The bound proteins were eluted and probed with the polyclonal antibodies against GST.
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FIGURE 5. Binding of GST-Cav-1-(61–101) to the gp41 peptides N36 and C34 and to the gp41 core formed by N36(L8)C34. Glutathione-Sepharose bead-conjugated GST was incubated with N36 (lane 2), C34 (lane 5), or N36(L8)C34 (lane 8), while the glutathione-Sepharose bead-conjugated GST-Cav-1-(61–101) was incubated with N36 (lane 3), C34 (lane 6), or N36(L8)C34 (lane 9). The bound peptides were eluted and analyzed by SDS-PAGE. As controls, the untreated peptides N36 (lane 4), C34 (lane 7), N36(L8)C34 (lane 10), GST (lane 11), and GST-Cav-1-(61–101) (lane 12), were run in SDS-PAGE under the same conditions. Lane 1 shows the protein molecular mass markers.

GST-Cav-1-(61–101) can interact with the gp41 expressed on the membranes of CHO-WT cells.

Subsequently, we transfected 293T cells with the plasmid of pcDNA3.0-plg-rsgp41 to express human IgG Fc-tagged rsgp41 (rs gp41-Fc) fusion protein in cytoplasm. GST-Cav-1-(61–101) and GST alone were added to the cell lysates, respectively. If GST-Cav-1-(61–101) binds to the endogenous rsgp41 expressed in the cytoplasm of 293T cells, these two proteins can be co-precipitated with Protein G-conjugated beads. GST fusion proteins in the eluates could be detected by anti-GST antibodies in Western blotting. For comparison, an identical amount of GST was used as a control. As shown in Fig. 4A, the input GST (lane 1) showed one band, whereas the input GST-Cav-1-(61–101) (lane 2) exhibited two bands, the lower one was GST and the upper one corresponded to GST-Cav-1-(61–101). GST-Cav-1-(61–101) could not be co-precipitated by rsgp41-Fc (lane 3), whereas GST-Cav-1-(61–101) was well precipitated due to its binding to rsgp41-Fc (lane 4). These results suggest that Cav-1-(61–101) can also bind to endogenous rsgp41 expressed in the cytoplasm of 293T cells.

The binding ability of GST-Cav-1-(61–101) to N36(L8)C34, N36, and C34 was also detected in a pull-down assay. The peptides bound to GST-Cav-1-(61–101) beads were eluted, analyzed with SDS-PAGE, and inspected by silver stain. As shown in Fig. 5, GST-Cav-1-(61–101) could strongly bind to N36(L8)C34 (lane 9) but was unable to bind to either N36 (lane 3) or C34 (lane 6). As a control, GST itself could not bind to N36 (lane 2), C34 (lane 5), or N36(L8)C34 (lane 8). These results suggest that the cytosolic membrane-proximal region of Cav-1 can interact with the gp41 core but not with the isolated NHR and CHR fragments.

Identification of the gp41 Core-binding Motif in the Scaffolding Domain of Cav-1—Above results confirmed that gp41 core domain could interact with the Cav-1 cytosolic membrane-proximal region. But it is unclear which site in this region is responsible for the interaction. We observed that the cytosolic membrane-proximal region contained a scaffolding domain (aa 82–101), which serves as the binding site for several proteins (21) and involves in the binding of Cav-1 to the HIV-1 gp41 (18). Furthermore, we noticed that the scaffolding domain did not contain the \( \Phi X \Phi XXX \Phi \) motif, but possessed the \( \Phi XXX \Phi \) motif (aa 92–99: FTVTKYWF). We thus suspected that the HIV-1 gp41 core might bind to the \( \Phi XXX \Phi \) motif. To prove this hypothesis, we constructed the following plasmids: pcDNA3.0-EGFP-Cav-1-(82–101) and pcDNA3.0-EGFP-Cav-1-(61–101) encoding the EGFP-tagged Cav-1-(82–101) and Cav-1-(61–101), respectively, both of which contain the \( \Phi XXX \Phi \) motif, pcDNA3.0-EGFP-Cav-1-(61–88) encoding the EGFP-tagged Cav-1-(61–88), which did not contain the \( \Phi XXX \Phi \) motif, and pcDNA3.0-plg-rsgp41, which expresses the human IgG Fc-tagged rsgp41. These plasmids were transfected into 293T cells. The 293T cells transfected with an empty EGFP vector or pcDNA3.0-EGFP-Cav-1-(82–101) showed bands with the respective expected molecular sizes. To determine the potential interaction between the scaffolding domain of Cav-1 and rsgp41 in 293T cells, the plasmids pcDNA3.0-EGFP-Cav-1-(82–101) and pcDNA3.0-plg-rsgp41...
were co-transfected into 293T cells. Similarly, the 293T cells co-transfected with pcDNA3.0-pIg-rsgp41 plus EGFP-Cav-1-(61–101), EGFP-Cav-1-(61–88), empty EGFP vector, or without transfection were used as controls. The cell lysates were immunoprecipitated with Protein G beads. The bound proteins were eluted and analyzed by SDS-PAGE and Western blotting with anti-EGFP antibodies. As shown in Fig. 6B, no protein bands were revealed in lanes 6 and 7 where the lysate of wild-type 293T cells and those transfected with empty EGFP vector, respectively, were loaded. A clear band with the expected molecular size of EGFP-Cav-1-(82–101) was shown in lane 8. The positive control EGFP-Cav-1-(61–101) also showed a band in the corresponding site in lane 9, whereas the negative control EGFP-Cav-1-(61–88) showed no band (lane 10). These results suggest that 293T cell-expressed EGFP-tagged Cav-1-(82–101) can bind with the IgG Fc-tagged rsgp41, which is precipitated by Protein G beads.

To determine whether the scaffolding domain of Cav-1 also binds to the gp41 core, we expressed GST-Cav-1-(82–101) and GST-Cav-1-(61–88) in E. coli and purified it with glutathione-Sepharose 4B affinity column. GST-Cav-1-(61–88) was also expressed and purified as a control to exclude the possibility that the gp41 core might bind to the sequence outside the ΦXXXΦ motif in Cav-1-(61–101). After separation with SDS-PAGE, a band with the expected molecular size of GST-Cav-1-(82–101) (~28 kDa) was revealed (Fig. 7A). Binding of the scaffolding domain of Cav-1 to the 6-HB of gp41 was analyzed by a pull-down assay using GST-Cav-1-(82–101) coupled to glutathione-Sepharose beads and Western blotting using anti-N36/C34 antibody as a probe. As shown in Fig. 7B, GST-Cav-1-(82–101) (lane 1), like GST-Cav-1-(61–101) (lane 2), strongly bound to N36(L8)C34, whereas the controls GST (lane 3) and GST-Cav-1-(61–88) (lane 4) did not interact with the gp41 6-HB directly. These results confirm that the scaffolding domain of Cav-1 can bind to the gp41 core.

We measured the binding affinity of GST-Cav-1-(82–101) to N36(L8)C34 using SPR assay. GST-Cav-1-(82–101) bound to N36(L8)C34 with $K_d = 1.18 \times 10^{-7}$ M (Table 2). GST-Cav-1-(61–101) also bound to N36(L8)C34, whereas the GST-Cav-1-(61–88) showed no binding activity to the N36(L8)C34 (Fig. 8A).

In the competition ELISA, JCH-1 blocked the binding of N36(L8)C34 to the immobilized Cav-1-(61–101) ($IC_{50} = 2.906 \pm 0.024 \mu g/ml$) or Cav-1-(82–101) ($IC_{50} = 3.07 \pm 0.048 \mu g/ml$) in a dose-dependent manner, whereas the control peptide (NM2) showed no inhibitory activity (Fig. 8B). These data suggest that the peptide JCH-1 expressing the ΦXXXΦ motif may have common binding sites on the 6-HB bundle of the gp41 core with Cav-1-(61–101) and Cav-1-(82–101).

**DISCUSSION**

Like other viral fusion proteins, the HIV-1 Env transmembrane subunit gp41 plays a critical role in membrane fusion, an essential step for virus entry into the target cell. However, gp41 may also mediate viral entry through alternative pathways, e.g. endocytosis, under the assistance of some cellular proteins. In the present study, we intended to identify a motif in a cellular protein that binds to gp41, especially the 6-HB formed by the viral NHR and CHR, because it is a critical structure mediating the membrane fusion process. Using the polypeptide N36(L8)C34 as a model of gp41 core to screen a 12-mer phage display library, we identified a gp41 core-binding phage clone, which displayed a peptide sequence that may be responsible for binding with gp41 core. We synthesized a peptide containing this sequence and demonstrated that this peptide specifically bound to the 6-HB core of gp41 and exhibited inhibitory activity against HIV-1-mediated cell-cell fusion (Fig. 3), suggesting the potential of designing...
peptides with gp41-core binding motif(s) as HIV-1 fusion inhibitors. After analyzing the peptide sequence, we noticed that this sequence contains a motif, \( \Phi XXX \Phi XX \Phi \), which is similar to that presented in the cytosolic membrane-proximal region (aa 61–101) of Cav-1.

Cav-1 is a major integral protein in the membrane of caveolae, a flask-shaped invagination of plasma membrane with endocytotic properties (Fig. 9). The caveolar membrane consists of detergent-resistant glycosphingolipid-rich membrane microdomains, termed lipid rafts (22, 23). A number of pathogens including viruses may utilize lipid rafts to exert their pathogenic effects (24). Cav-1 is essential for caveolae, a flask-shaped invagination of plasma membrane with endocytic properties (Fig. 9). The caveolar membrane microdomain (aa 82–101), which is responsible for oligomerization and for binding a number of cellular proteins involving in signal transduction (21). Notably, this domain contains a putative gp41 core-binding motif (\( \Phi XXX \Phi XX \Phi \)) and is located in the juxtamembrane region. We postulated that the Cav-1 scaffolding domain may be responsible for interaction of Cav-1 with the gp41 core.

This assumption was proven to be valid by the experimental data in this study: (a) the fusion protein, Cav-1-(61–101), which overlaps the sequences of cytosolic membrane-proximal region and contains the scaffolding domain of Cav-1, could capture gp41 expressed on cell membranes and inside cytoplasm in pull-down assays (Fig. 4); (b) the fusion protein, Cav-1-(82–101), which covers the Cav-1 scaffolding domain and the \( \Phi XXX \Phi XX \Phi \) motif, was able to precipitate the endogenous gp41 ectodomain (Fig. 6), whereas the fusion protein Cav-1-(61–88) that does not contain the \( \Phi XXX \Phi XX \Phi \) motif showed no binding ability to the gp41; and (c) both Cav-1-(61–101) and Cav-1-(82–101) strongly bound to the polypeptide N36(L8)C34 that forms the stable 6-HB core of gp41 in physiological solution (Figs. 5, 7, and 8). The interaction between the HIV-1 gp41 core and the Cav-1 scaffolding domain may be responsible for HIV-1 entry into the target cell via endocytosis.

HIV-1 has been reported to use lipid rafts and caveolae as the endocytosis route to enter the target cells (25). Destabilization of lipid raft patches in the lymphocyte plasma membrane may facilitate HIV-1 penetration of lymphocytes (26). Hovanessian et al. (18) demonstrated that the native HIV-1 gp41 molecules bound to Cav-1, through a hydrophobic motif (\( \Phi XXX \Phi XX \Phi \): WNNMTWMEW(631)), spanning from the end of the loop to the last four amino acid residues at the N terminus of the gp41 CHR region, to form a complex in the HIV-1-infected cells. The N-terminal end of C34 in the N36(L8)C34 peptide contains a part of this motif (\( \Phi XX \Phi \): WMEW(631)). Therefore, besides the native gp41 molecule, the gp41 core can also bind to Cav-1 through the interaction between the \( \Phi XXX \Phi XX \Phi \) motif in the Cav-1 scaffolding domain and a section of the \( \Phi XX \Phi \) motif in the N-terminal region of C34 (C-helix) located at the external surface of the 6-HB.

Coutet et al. (33) have identified two caveolin-1 binding motifs: \( \Phi \Phi XXX \Phi \Phi \) and \( \Phi XXX \Phi XX \Phi \). The peptide sequence of the phage clone gcb-1 and JCH-1 (WHWTSYLYG-WSS), although it does not have the \( \Phi XXX \Phi XX \Phi \) motif, contains the caveolin-1 binding motif \( \Phi \Phi XXX \Phi XX \Phi \) and its reversed version \( \Phi XXX \Phi XX \Phi \). By searching the protein database, we found that, besides Cav-1, several other proteins, e.g.
**HIV-1 gp41 Core-binding Motif in Caveolin-1**

The HIV-1 gp41 core may not need the assistance of a cellular protein to form fusion pore for completion of the fusion process. However, the degraded fragments from the viral NHR and CHR regions, like the synthetic NHR- and CHR-peptides (3, 30, 31), may interact in a corresponding manner with the viral CHR and NHR regions to form heterologous 6-HBs, blocking the further process of membrane fusion. In addition, soluble CD4 molecules, which may be released from CD4+ cells, can bind to gp120 on virions to trigger gp41 core formation (20, 32). These virions thus lose fusion activity, but they may still be able to enter the target cells through endocytosis mediated by interaction of the gp41 core on the viral particles with the scaffolding domain of Cav-1 in the caveolar membranes (Fig. 9).

The present study provided the first evidence for a direct interaction between the 6-HB core of the HIV-1 gp41 and the scaffolding domain of Cav-1 and identified a putative gp41 core-binding motif in the Cav-1 scaffolding domain, which is responsible for oligomerization and binding sites for various proteins involving in signal transduction. Interaction of gp41 core with Cav-1 may facilitate the fusion pore formation or mediate endocytosis of HIV-1 particles. Further characterization of the gp41 core-binding motif and study of the gp41 core-Cav-1 interaction will provide important information for understanding the fusogenicity of the HIV-1 gp41 and the pathogenesis of HIV-1 infection and for the rational design of novel HIV-1 entry inhibitors.

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**FIGURE 9. The potential outcomes of the HIV-1 gp41 core binding to the scaffolding domain of Cav-1.** Caveolae are flask-shaped invaginations of the detergent-resistant membranes (also named lipid rafts) of cells, characterized by high content in glycosphingolipids and cholesterol. Cav-1 is the main protein in lipid rafts and essential for caveolae formation. Its N and C termini are located in plasma, whereas its middle part containing the scaffolding domain is located in the membrane, which is responsible for oligomerization and interaction with a number of signal molecules and cellular proteins, e.g. CD26, a regulatory molecule of T cell activation. HIV-1 Env consists of gp120/gp41. Once gp120 binds with CD4 and a co-receptor on the host cell, gp41 changes its conformation, leading to insertion of the fusion peptide into the cell membrane and association of the NHR and CHR regions to form a 6-HB (gp41 core), which may participate in fusion pore formation, possibly with the assistance of Cav-1. Alternatively, the gp41 core on the surface of the HIV-1 virions may bind to Cav-1 in the caveolar membranes (or lipid rafts), resulting in endocytosis of the viral particles.
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