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Identification of a conserved domain of the HIV-1 transmembrane protein gp41 which interacts with cholesteryl groups

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Abstract

A soluble form of the HIV-1 envelope glycoprotein gp160 devoid of the transmembrane anchor domain was found to bind to cholesteryl-hemisuccinate agarose. The external subunit gp120 failed to bind to the resin, suggesting that the site responsible for the binding to cholesterol was located in the transmembrane protein gp41.

We constructed a series of maltose binding protein (MBP) fusion proteins representing overlapping fragments of the gp41 molecule and we studied their capacity to bind to cholesteryl beads. The domain responsible for binding to cholesterol was localised within the residues 668 to 684 immediately adjacent to the membrane spanning domain. We identified a short sequence (LWYIK, aa 678–683) comparable to the cholesterol interaction amino acid consensus pattern published by Li and Papadopoulos [Endocrinology 139 (1998) 4991]. We demonstrated that the sequence LWYIK synthesized fused to the MBP was able to bind to cholesteryl groups. A synthetic peptide containing the sequence LWYIK was found to inhibit the interaction between cholesteryl beads and MBP44, an MBP fusion HIV-1 envelope protein that contains the putative cholesterol binding domain. Human sera obtained from HIV-1 seropositive patients did not react in ELISA to the LWYIK sequence, suggesting that this region is not exposed to the immune system. The biological significance of the interaction between gp41 and cholesterol is discussed.

Keywords: HIV-1; HIV-1 envelope protein; Cholesterol; Maltose binding protein

1. Introduction

The HIV-1 envelope protein is synthesized from a precursor gp160. During its transport to the cell surface, a fraction of gp160 is cleaved to gp120–gp41. The external gp120 is responsible for the attachment to the cellular receptor and coreceptor(s), whereas the transmembrane protein gp41 is responsible for fusion of the viral envelope with the plasma membrane of the target cells. Membrane fusion requires the cleavage of the inactive precursor gp160 and is thought to be mediated by the hydrophobic N-terminus of gp41. Previous studies have demonstrated a link between fusion and lipid composition for several enveloped viruses. Roos et al. [2] have reported that the manipulation of cellular lipid content modified the susceptibility of cells to viral fusion. Semliki Forest Virus has been demonstrated to require cholesterol, a major component of mammalian cell plasma membrane for membrane fusion and exit [3]. Studies have shown that the fusion peptide of Semliki Forest Virus and the F1 subunit of the fusion protein of Sendai virus bind to cholesterol [4,5]. Inhibitors of cholesterol synthesis have been shown to inhibit the syncytia formation induced by Measles virus [6]. Several observations suggest that the HIV-1 envelope glycoprotein may also interact with cholesterol. Membranes of HIV and HIV-infected cells are rich in cholesterol and the cholesterol-to-phospholipid molar ratio of HIV-1 envelope is 2.5 times that of its host cell membrane [7]. Inhibitors of cholesterol synthesis also inhibit cell fusion formation induced by HIV-1 [8]. Drugs that extract cholesterol from the cellular membrane exert an anti-HIV effect in vitro [9–11]. Recent studies suggest

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that HIV-1 uses cholesterol and sphingolipids during budding [12]. The objective of this study was to investigate the interaction of cholesteryl groups with the HIV-1 envelope protein. We have determined that a small region immediately adjacent to the membrane spanning domain of gp41 was sufficient to confer interaction with cholesteryl groups.

2. Materials and methods

2.1. Chemicals

Cholesteryl-hemisuccinate agarose (1 μmol cholesteryl hemisuccinate per milliliter of packed gel), concanavalin A (Con A) sepharose 4B (16 mg lectin per milliliter of packed gel), calmodulin (CaM) agarose (1.7 mg calmodulin per milliliter of packed gel) and cholic acid agarose (5 μmol cholic acid per milliliter packed gel) were from Sigma. Peptides 2032 (aa 675–685, HIV-1 MN, DITNWLWYIKI), 4767 (aa 671–690, HIV-1 89.6P, FDITNWLWYIRLFIMIVGGL) and 2029 (aa 641–660, HIV-1 MN, SLIYSLLEKSQTQQEKNEQE) were obtained from the NIH AIDS Research and Reference Reagent Program. Peptides EVA 798.20 (aa 701–720, SIV, ILLRIVIYIV QMLAKLRQGY) was obtained through the Medical Research Council AIDS Reagent Project. Purified maltose binding protein (MBP) was from New England BioLabs.

2.2. Vaccinia virus (VV) recombinants

VV recombinants, VVTG1132 expressing gp120LAI and VVTG5156 expressing a soluble chimeric envelope glycoprotein containing gp41LAI and gp120MN, were obtained from Dr. M.-P. Kieny [13]. VVTG5156, which is a derivative of VVTG1163 [13] in which the HIV LAI gp120 coding sequence has been replaced by HIV-1MN counterpart, contains an envelope gene modified by site-directed mutagenesis to destroy the gp120–gp41 cleavage site, to remove the transmembrane anchor domain of gp41 and to truncate gp41 at aa 29 into the intracytoplasmic domain.

2.3. Metabolic labelling with ^{35}S-methionine/cysteine

HeLaT4 cells (HeLa cells stably expressing CD4) were maintained in RPMI-1640 medium supplemented with 10% foetal bovine serum, glutamine, 10 mM HEPES and penicillin/streptomycin in 25-cm^3 tissue culture flasks. For radiolabelling of HIV-1 envelope protein expressed from VV recombinants, confluent HeLaT4 cells were infected with VV (5 PFU/cell) and 16 h later labelled for 4 h with ^{35}S-methionine/cysteine (TRANS ^{35}S-LABEL, ICN Radiochemicals) (100 μCi/ml) in methionine/cysteine deficient medium. The medium (2 ml) was added 30 min before the addition of radioactivity. The supernatants that contained the labelled secreted proteins were stored at −20 °C.

2.4. Construction of MBP fusion HIV-1 proteins

An isopropyl-beta-D-thiogalactopyranoside (IPTG) inducible expression plasmid pMAL-c2E (New England BioLabs) was used to generate recombinant MBP fusion proteins containing fragments of the HIV-1 envelope protein. The foreign sequence was inserted in frame, downstream of the *Escherichia coli malE* gene, which encodes MBP. The plasmids containing the wild-type HIV-1 envelope glycoprotein genes used to generate the constructs have been previously described [14].

The plasmid pMBP35, which expresses the amino acid sequence 463–619 of 92/UG/024 HIV-1 isolate, was obtained by inserting *Spe*I (nt1365)–*Xba*I (nt1835) fragment from p92/UG/024 (ARP2008) into pMAL-c2E.

The plasmid pMBP39, which expresses the amino acid sequence 542–641 of HIV-1 LAI isolate, was obtained by inserting a 286 bp *Hae*III (nt7835)–*Hin*dIII (nt8121) fragment from pNL4-3 into pMAL-c2E.

The plasmid pMBP44, which expresses the amino acid sequence 618–684 of HIV-1 92/BR/025.9 isolate, was obtained by inserting a 202 bp *Bgl*II (nt1824)–*Ssp*I (nt2026) fragment from p92/BR/025.9 (ARP239-1) into pMAL-c2E.

The plasmid pMBP42, which expresses the amino acid sequence 668–801 of HIV-1 GB8 isolate, resulted from the insertion of an *Apo*I (nt1980)–*Pst*I (nt2381) fragment from pGB8.C4 (ARP210) into pMAL-c2E.

The plasmids pMBP31 and pMBP45, which express the amino acid sequences 583–856 and 752–856 of HIV-1 92/UG/975 isolate, respectively, were obtained by inserting the *Xba*I (nt1712)–*Pst*I (nt2589) and *Xmn*I (nt2214)–*Pst*I (nt2589) fragments from p92/UG/975 (ARP2007) into pMAL-c2E, respectively.

The plasmid pMBP38, which expresses the amino acid sequence 640–752 of HIV-1 LAI isolate, was obtained by inserting a 344 bp *Hin*dIII (nt8121)–*Bam*HI (nt8465) fragment from pNL4-3 into pMAL-c2E.

The construct MBP-LWYIK, which includes amino acids 679–683, was obtained by introducing a synthetic DNA fragment encoding the amino acids LWYIK followed by a stop codon at the end of the *malE* gene so that eight arginine residues separate the MBP from the peptide sequence. This plasmid was constructed by a two-step PCR amplification using the primers mal51 (5′-CTGTGGTATATAAAATGAAACCTCGGGGATGACGATGACAAGG-3′) and mal31 (5′-TCATTTTATATACCACAGGTTATTGTTATTGTTGTTGTTCGAGCTCGAA-3′) for the insertion, and mal30 (5′-GGTAACGCCAGGGTTTTCCCAGTC-3′) and mal50 (5′-GCTTACCCGATCGCTGTTGAAGCGTTA-3′) as flanking primers. The 1-kb fragment that was generated was purified and inserted into pMAL-c2E by *Bgl*II–*Hin*dIII digestion.

All the inserted sequences were verified by sequencing. The numbering of residues is based on the sequence of the gp160 of the HIV-1 HXB2.

2.5. Purification of MBP fusion HIV-1 proteins

Protein expression was induced by IPTG as recommended by the manufacturer. Cells were harvested by centrifugation and resuspended in column buffer (10 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). Proteins were released in the supernatant by cell sonication. After centrifugation, the MBP fusion protein purification was done by amylose affinity chromatography according to the manufacturer's protocols or by using the method published by Srinivasan and Bell [15]. Purified MBP proteins were separated by SDS-PAGE and detected by Western blotting and ELISA using an anti-MBP rabbit antiserum (New England BioLabs) and/or specific monoclonal antibodies.

2.6. Iodination of MBP44

MBP44 in column buffer was iodinated with ^{125}I (as Na^{125}I Amersham) according to the protocol described by Helmkamp et al. [16]. After labelling, MBP44 was purified on an amylose resin column by affinity chromatography.

2.7. Interaction of ^{35}S-labelled HIV-1 glycoproteins with resins

Supernatants containing labelled HIV-1 envelope proteins were diluted in 1 ml PBS and added to the resin (100 μl). The resin suspension was incubated 2 h at room temperature with constant mixing. After centrifugation, the supernatant was discarded and the pellet extensively washed with PBS. The proteins were dissociated from the resin by boiling for 1 min in sampling buffer (65 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) in presence of 1% 2-mercaptoethanol. The ^{35}S-HIV-1 envelope proteins released from the resin were analyzed by 7% SDS-PAGE and visualized by autoradiography.

2.8. Interaction of MBP fusion proteins with cholesteryl-hemisuccinate agarose

MBP fusion proteins were diluted in 1 ml PBS and added to the resin (100 μl). The resin suspension was processed as indicated above. After dissociation from the resin by boiling, the proteins were analyzed by 8% SDS-PAGE. The MBP fusion proteins were transferred onto nitrocellulose. The membrane was blocked for 1 h with PBS containing 5% skim milk and incubated overnight at 4 °C with rabbit anti-MBP antiserum (New England Biolabs). After washing with PBS, bound antibody was probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma) and developed with 3,3-diaminobenzidine (DAB) peroxidase substrate (Sigma Fast) or processed with the ECL Western Blotting Kit (Amersham).

2.9. ELISA methods

For peptide ELISA, microtiter plates were coated with peptides 2032 or 4767 (0.5 μg/well) in 0.1 M sodium carbonate buffer (pH 9.6) overnight at 4 °C. The plates were washed with TBS (144 mM NaCl, 25 mM Tris–HCl, pH 7.5) containing 0.5% Tween 20 and blocked with the same buffer plus 2% BSA for 1 h at 37 °C. After washing, 100 μl of serum (diluted 1:50 in TBS) (obtained from the University Hospital of Saint-Etienne and from the University Hospital of Lyon) were added to the wells and incubated overnight at 4 °C. After washing, the detection was done by using successively horseradish peroxidase-conjugated goat anti-human IgG and *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma). The measurement of absorbance was read at 492 nm. The cutoff value was defined as the mean value of absorption of negative control samples plus 2 S.D.

For MBP-LWYIK ELISA, microtiter plates were coated with 150 μl 0.5% dextrin in TBS overnight at 4 °C. The plates were washed with TBS containing 0.5% Tween 20 and blocked with the same buffer plus 2% BSA for 1 h at 37 °C. Sonicated bacterial extract containing MBP-LWYIK was centrifuged and 100 μl of the supernatant (500 μg protein/ml) was added to the wells for 3 h at 37 °C. After washing, 100 μl of serum (diluted 1:50) was added to the wells and incubated overnight at 4 °C. The detection procedure was the same as that described above.

3. Results

3.1. Interaction of ^{35}S-HIV-1 envelope glycoproteins with cholesteryl-hemisuccinate agarose

To examine the putative interaction of the HIV-1-envelope with cholesterol, we studied the retention of the env protein metabolically labelled with TRAN ^{35}S on cholesteryl-hemisuccinate agarose. Con A sepharose 4B and CaM agarose were used as positive controls. Previous studies have demonstrated that Con A, a mannose-specific lectin, interacts strongly with gp120 and that CaM, a EF-hand protein, binds with high affinity to the cytoplasmic tail of the gp41 [17–19]. The cells were infected with recombinant vaccinia virus VVTG5156, which expresses a soluble and uncleaved form of HIV-1 gp160 devoid of the membrane spanning domain. The cells were labelled with ^{35}S-methionine/cysteine as described in Materials and methods. Equal fractions of the supernatant containing secreted ^{35}S-gp160$_{5156}$ were incubated in the presence of the resins. As shown in Fig. 1A, the ^{35}S-gp160$_{5156}$ bound to cholesteryl-hemisuccinate agarose with the same affinity as to CaM agarose and Con A sepharose 4B. The ^{35}S-gp160$_{5156}$ bound with a much lower affinity to heparin agarose and to the hydrophobic resin cholic agarose even if cholic acid and cholesterol are structurally close. Heparin, a highly charged anionic glycosylaminoglycan, is known to inhibit the HIV-1-induced cell

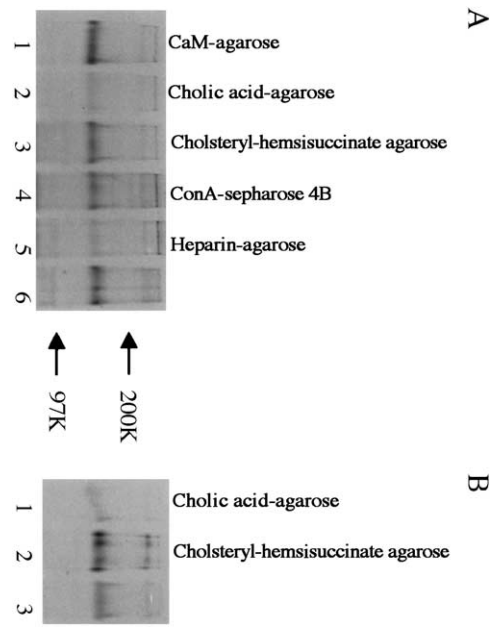

Fig. 1. Interaction of ^{35}S-recombinant HIV-1 envelope glycoprotein with cholesteryl-hemisuccinate agarose. (A) Interaction of ^{35}S-gp160$_{5156}$ with resins: lane 1, CaM-agarose; lane 2, cholic acid agarose; lane 3, cholesteryl-hemisuccinate agarose; lane 4, Con A-sepharose 4B; lane 5, heparin-agarose. Lane 6 shows ^{35}S-gp160$_{5156}$ immunoprecipitated with MAb 27 and adsorbed onto Sepharose-protein A. The samples were run on 7% SDS-PAGE under reducing conditions. The positions of the radiolabelled molecular weight markers are shown on the right. (B) Interaction of ^{35}S-gp160$_{5156}$ with resins: lane 1, cholic acid agarose; lane 2, cholesteryl-hemisuccinate agarose. Lane 3 shows ^{35}S-gp160$_{5156}$ immunoprecipitated with MAb 27 and adsorbed onto Sepharose-protein A. The samples were run on 7% SDS-PAGE under nonreducing conditions.

fusion by electrostatic interaction with the gp120 subunit [20]. In Fig. 1B, the samples have been analyzed under nonreducing conditions. We observed that monomeric and dimeric forms of ^{35}S-gp160$_{5156}$ interacted with cholesteryl-beads. To show the position of ^{35}S-gp160$_{5156}$, an aliquot of the supernatant of vaccinia-infected cells was immunoprecipitated with anti-gp41 MAb 27 and Sepharose-protein A (Fig. 1A, lane 6; B, lane 3). To determine if the cholesterol binding domain was in the gp120 subunit, ^{35}S-labelled gp120 (secreted from cells infected with VVTG1132 recombinant) was incubated with resins. Con A-derivatized resin was used as a positive control and CaM agarose as a negative control. There is no CaM-binding domain in gp120 subunit [19]. ^{35}S-gp120HIV-1 LAI bound with a high affinity to Con A derivatized resin but not to the cholesteryl-hemisuccinate and CaM derivatized resins (not shown).

3.2. Interaction of MBP-fusion proteins representing regions of gp41 with cholesteryl-hemisuccinate agarose

To characterise the region of gp41 which binds to cholesterol, we used a series of recombinant proteins that represent the whole gp41 molecule. Envelope gene fragments were ligated into the pMAL-c2E plasmid and expressed in *E. coli*. Sonicated bacterial extracts containing HIV-1 recombinant MBP protein were centrifuged and an aliquot of each supernatant was incubated with immobilized cholesterol. The complexes were washed in PBS, denatured in sampling buffer and then an aliquot of each supernatant was analyzed by SDS-PAGE. The MBP protein was detected by Western blotting with an anti-MBP antiserum and DAB substrate. As shown in Fig. 2, the construct MBP35 (aa 463–619), which contains the fusogenic sequence of gp41, the construct MBP45 (aa 752–856) which contains a fragment of the cytosolic domain of gp41, and the construct MBP39 (aa 542–641) which contains an immunodominant epitope of gp41 failed to bind to cholesteryl groups. The constructs MBP42 (aa 668–801), MBP38 (aa 640–752) and MBP31 (aa 583–856) interacted with immobilized cholesterol. In the experiment illustrated by Fig. 3, increasing concentrations of purified MBP44 (aa 618–684) (panel A) and MBP38 (panel B) were incubated with the same amount of cholesteryl-beads. Proteins were transferred to nitrocellulose, probed with an anti-MBP antiserum and visualized with the ECL detection system.

Purified MBP (43 K) was used as a molecular weight marker and to evaluate the detection system (Fig. 3A, lanes 1 and 2; B, lane 1; C, lanes 1 and 2). Purified MBP did not bind to cholesteryl beads (not shown). MBP44 exhibited monomeric and dimeric forms and both forms interacted with cholesteryl-beads. We observed that comparable amounts of MBP44 and MBP38 interacted with cholesteryl-agarose. To determine the region of gp41 involved in cholesterol binding recombinant envelope protein sequences were aligned (Fig. 4). Cholesteryl groups bound to a region spanned by the constructs MBP44 and MBP42. MBP44 and MBP42 overlap by 17 amino acids.

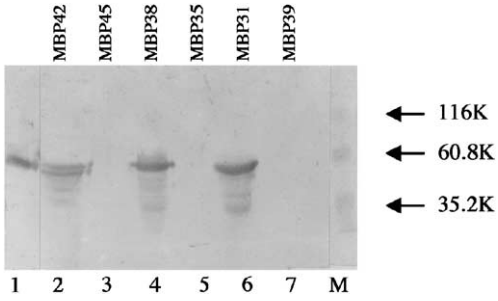

Fig. 2. Interaction of sonicated bacterial extract containing MBP fusion proteins representing fragments of HIV-1 envelope protein with cholesteryl-hemisuccinate agarose. Clarified sonicated bacterial extract (300 μl) containing MBP42 (lane 2), MBP45 (lane 3), MBP38 (lane 4), MBP35 (lane 5), MBP31 (lane 6) and MBP39 (lane 7) were incubated with cholesteryl-hemisuccinate agarose (100 μl). The proteins were released from the beads and analyzed by SDS-PAGE as indicated in Materials and methods. After Western blotting, the membranes were incubated in the presence of a rabbit anti-MBP antiserum. Bound antibodies were revealed with an anti-rabbit IgG peroxidase conjugate and DAB peroxidase substrate. Lane 1 shows purified MBP obtained from New England BioLabs (100 ng). The lane M contains molecular weight markers.

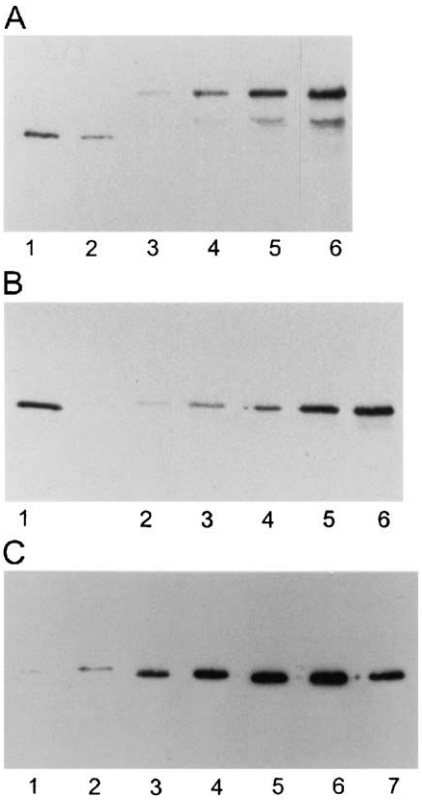

Fig. 3. Interaction of purified MBP-recombinant HIV-1 envelope proteins with cholesteryl-hemisuccinate agarose. (A) Interaction of 25 ng (lane 3), 50 ng (lane 4), 75 ng (lane 5) and 125 ng (lane 6) of MBP44 with resin (50 μl). The same aliquots of sample (25 μl) were loaded in each well. Lanes 1 and 2 show 10 and 5 ng of purified MBP from New England BioLabs, respectively. (B) Interaction of 25 ng (lane 2), 50 ng (lane 3), 75 ng (lane 4), 100 ng (lane 5) and 125 ng (lane 6) of MBP38 with resin (50 μl). The same aliquots of sample (25 μl) were loaded in each well. Lane 1 shows purified MBP from New England BioLabs (20 ng). (C) Interaction of 200 ng (lane 3), 400 ng (lane 4), 600 ng (lane 5) and 900 ng (lane 6) of MBP-LWYIK with resin (100 μl). The same aliquots of sample (10 μl) were loaded in each well. Lanes 1 and 2 show 5 and 10 ng of purified MBP from New England BioLabs, respectively. Lane 7 shows an aliquot of purified MBP-LWYIK prior to interaction with the resin. In A, B and C, the samples were run on 8% SDS-PAGE under reducing conditions. After Western blotting, the proteins were visualized with the ECL detection system. The signal strength was estimated after 15–20 s exposure.

3.3. Interaction of MBP-fusion protein representing the sequence LWYIK of gp41 with cholesteryl-hemisuccinate agarose

To more closely characterise the location of the cholesterol binding site, we looked in the sequence 668–684 of HIV-1 envelope glycoprotein for the presence of the cholesterol recognition/interaction amino acid consensus pattern identified by Li and Papadopoulos [1]. These authors described a consensus pattern for proteins that bind cholesterol as -L/V-$(X)_{1-5}$-Y-$(X)_{1-5}$-R/K-. This pattern was found in the sequences LWYIK or LWYIR immediately adjacent to the membrane-spanning domain of gp41. To investigate whether the binding was dependent on amino acids LWYIK, a construct expressing the motif LWYIK was realised by PCR. Increasing concentrations of purified MBP-LWYIK were incubated with the same amount of cholesteryl-beads. Proteins were transferred to nitrocellulose, probed with an anti-MBP antiserum and visualized with the ECL detection system. As shown in Fig. 3C, MBP-LWYIK interacted with immobilized cholesterol. By Western blotting and detection with an anti-MBP antiserum and DAB substrate, we estimated that approximately 700 ng of MBP-LWYIK were able to bind to 100 μl of packed gel (not shown).

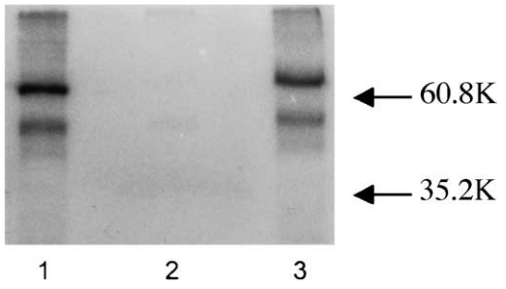

Fig. 5. Inhibition of the interaction between ^{125}I-MBP44 and cholesteryl-hemisuccinate agarose by synthetic peptide 2032. Purified ^{125}I-MBP44 was incubated in the presence (lane 2) or absence (lane 3) of peptide 2032. Peptide 2032 (10 μg) was diluted in 1 ml PBS and incubated for 2 h with cholesteryl-hemisuccinate agarose (100 μl). ^{125}I-MBP44 (700 ng) was added to the resin suspension and then the mixture was incubated for a further 2 h. The proteins were released from the beads and analyzed by SDS-PAGE as described in Materials and methods. The gel was dried and exposed to Bio Max Light-1 film (Kodak). Lane 1 shows an aliquot of purified ^{125}I-MBP44 prior to interaction with the resin. The migration positions of molecular weight markers are indicated.

3.4. Effects of synthetic peptides on the interaction ^{125}I-MBP44/cholesteryl-beads

We investigated the effects of the following peptides on the interaction between ^{125}I-MBP44 and cholesteryl-beads: 2032 representing the region that immediately precedes the transmembrane anchor and contains the

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MBP31 (583-856)    ...QQEKNEQDLLALDKWTSLWSWFSI SNWLWYIRIFIMIVGGLIGLRIIFAV...
MBP38 (640-752)    ...QQEKNEQELLELDKWASLWNWFNITNWLWYIKLFIMIVGGLVGLRIVFAV...
MBP42 (668-801)    NLWNWFDITNWLWYIKIFIMIIGGLIGLRIIFAV...
MBP44 (618-684)    ...QQEKNEQDLLALDKWQNLWTWFGITNWLWYIKI
                                  |                  |
                                 668                684
```

Fig. 4. Amino acid alignment of MBP constructs interacting with cholesteryl-hemisuccinate agarose. Shared sequences between these proteins are indicated.

LWYIK sequence, EVA 798.20 representing the transmembrane anchor of SIV, and 2029 representing a fragment of MBP44. Cholesteryl beads were incubated in the presence of peptide and ^{125}I-MBP44.

As shown in Fig. 5, the interaction between cholesteryl beads and MBP44 was inhibited by peptide 2032. Synthetic peptides EVA 798.20 and 2029 did not inhibit the interaction between ^{125}I-MBP44 and cholesteryl beads (not shown).

3.5. Comparison of the cholesterol binding site LWYIK of the HIV-1 envelope protein with homologous regions of other retroviruses

As shown in Fig. 6, the HIV-1 envelope sequence LWYIK is highly conserved among HIV-1 isolates. The sequence contains three hydrophobic amino acids (L, W, I). The lysine residue (K), a positively charged amino acid, adjacent to the membrane-spanning domain can be replaced by arginine (R), another positively charged amino acid. The conserved lysine residue is separated from two aromatic residues, tryptophane (W) and tyrosine (Y) by an isoleucine residue (I). The sequence LWYIK is also found in the SIVcpzant strain envelope protein. For most of the SIV and HIV-2 strains, the putative motif responsible for binding to cholesterol consists of 6 amino acids. The pattern is L(AS/TS)WI(K/R) and is slightly different from that described for HIV-1. However, the sequence is also characterised by a positively charged amino acid (K/R) adjacent to the membrane spanning region and separated from an aromatic amino acid by the hydrophobic residue isoleucine.

HIV-1		
	92/BR025-9	WQNLWTWFGITNWLWYIK
	GB8.C4	WANLWNWFDITNWLWYIK
	NL4-3	WASLWNWFNITNWLWYIK
	MN	WASLWNWFDITNWLWYIK
	HXB2	WASLWNWFNITNWLWYIK
	92/UG024-2	WASLWNWFDITNWLWYIR
SIV		
	SIVcpzant	WSSLWNWFDITQWLWYIK
	SIVcpz (Q88004)	LNSWDVFGNWFDLASWIK
	SIVcpz	LNSWDVFGNWFDLASWIR
	SIVmac251	LNSWDVFGNWFDLASWIK
	SIVagm	LNSWDVFG NWFDLASWIK
	SIVmac	LNSWDVFGNWFDLTSWIK
	SIVsm	LNSWDIFGNWFDLTSWIK
	SIVsm S4	LNSWDIFGNWFDLTSWIR
HIV-2		
	HIV2CBL24	LNSWDVFGNWFDLASWIK
	HIV-2ST	LNSWDVFGNWFDLTSWIK
	HIV2CBL21	LNSWDVFGNWFDLTSWIR

Fig. 6. Comparison of the conserved tryptophan-rich motif in the membrane-proximal region of gp41 with homologous regions of other retroviruses. The putative cholesterol binding sites are underlined.

3.6. Reactivity of sera of HIV-1 seropositive subjects to MBP-LWYIK

The reactivity of 27 sera collected from HIV-1 seropositive persons to MBP-LWYIK or to the peptides representing that region was determined by ELISA as described in Materials and methods. Ten sera collected from HIV-1 seronegative healthy persons were tested in parallel. None of the 27 sera tested was found to react with the sequence LWYIK or LWYIR (not shown).

4. Discussion

The studies described here provide evidence that HIV-1 gp160 binds to cholesteryl groups. The envelope protein binds to cholesteryl groups to a similar extent as that obtained with CaM- and Con A-derivatized resins. Con A-derivatized resins are usually used to purify gp120 molecules by affinity chromatography. The constructs MBP35, MBP39, MBP44, MBP42 and MBP45 contain hydrophobic domains [21,22]. Only MBP44 and MBP42 were found to bind to cholesteryl beads. This indicates that the interaction between MBP44 or MBP42 and cholesteryl groups cannot be explained simply by an interaction between a hydrophobic domain and a lipophilic compound.

We localised a cholesterol binding domain (sequence LWYIK) within the region immediately adjacent to the membrane-spanning domain of gp41. The sequence LWYIK has been found by comparison with the cholesterol recognition/interaction amino acid consensus pattern identified by Li and Papadopoulos [1] as -L/V-(X)$_{1-5}$-Y-(X)$_{1-5}$-R/K-. These authors have identified a cholesterol recognition amino acid motif on the carboxy-terminus of peripheral-type benzodiazepine receptor. This motif was common to various proteins known to interact with cholesterol [1]. In addition, they have demonstrated that replacement of Y by S or R by L completely abolished the ability of peripheral-type benzodiazepine receptor to take up cholesterol [1].

Previous studies have demonstrated that a stretch of 17 hydrophobic and uncharged amino acids immediately adjacent to the border of the membrane spanning domain of gp41 is required for envelope-mediated membrane fusion [23]. Helseth et al. [24] have demonstrated that a mutant with a change in the sequence LWYIK (replacement of the positively charged amino acid lysine by the nonpolar residue isoleucine) was defective for syncytium formation and replication even though the level of envelope glycoprotein on the cell or virion surface was comparable to that of the wild-type protein. Salzwedel et al. [23] found similar results by replacement of K with the sequence NSG (asparagine–serine–glycine) or with the nine membrane-primal residues of human erythrocyte decay-accelerating factor (DAF). Salzwedel et al. [23] also demonstrated that the deletion of LWYI had a significant effect on syncytium formation. These observations suggest strongly that inter-

action of LWYIK with cholesterol is involved in the fusion process. The fact that comparable amounts of MBP44 and MBP38 interact with cholesteryl-hemisuccinate-agarose suggests that the transmembrane region has a minor role in the binding to cholesterol. In agreement with this, we showed that the soluble and uncleaved form of the envelope protein gp160 deleted from the transmembrane region was able to bind cholesterol-derivatized resin agarose.

Asano and Asano [5] demonstrated that the hydrophobic side chain and the polar 3′ OH-group of cholesterol were not involved in the binding of the Sendai virus fusion protein with cholesterol. They postulated that the cholesterol binding to the fusion protein of Sendai virus was dependant of the cholesterol nucleus. In our studies, the polar hydroxyl group of cholesterol is esterified, suggesting a similar mechanism.

Similarities were found when the region immediately adjacent to the border of the membrane spanning domain of HIV-1 gp41 was compared to the envelope proteins of other retroviruses: the number of hydrophobic residues, the presence of a positively charged amino acid (K/R) adjacent to the membrane spanning region and separated from benzenic amino acid(s) by an isoleucine residue. This suggests that residues with aromatic ring and positively charged amino acid are particularly important in the motif.

In many of the SIV sequences, the Y residue, an aromatic amino acid, found in the putative cholesterol binding motif described by Li and Papadopoulos is replaced by W, another aromatic amino acid. In addition, Y and W belong to the same group of amino acids when classified by the chemical characteristics of their side groups (neutral–polar).

Sera collected from HIV-1 seropositive persons did not react with the sequence LWYIK, suggesting that the region adjacent to the membrane-spanning domain, hydrophobic, is poorly immunogenic or not exposed to the immune system.

The incorporation of HIV-1 envelope glycoproteins into liposomes has been demonstrated to preserve the native trimeric structure of the glycoprotein [25]. The presence of cholesterol in the liposomal solution may explain the capacity of liposomes to stabilize the HIV-1 envelope glycoproteins.

Cholesterol, sphingolipids and glycolipids are involved in the formation of cellular raft domains. Recent reports suggest that HIV-1 may use lipid rafts during budding [12]. The interaction of cholesterol with gp41 may be critical for localization of the HIV-1 envelope glycoprotein to lipid rafts.

Finally, it is probable that the interaction between cholesterol and the region immediately adjacent to the membrane spanning domain plays a major role in the fusion process. However, further studies are necessary to determine if that interaction is involved in other steps of the virus life cycle.

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