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HIV-1 neutralizing antibodies elicited by the candidate CBD1 epitope vaccine react with the conserved caveolin-1 binding motif of viral glycoprotein gp41

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Abstract

To date, candidate HIV-1 vaccines that have been tested in clinical trials have failed to induce broadly neutralizing activities and/or antibodies that inhibit infection by primary isolates of HIV-1. We recently identified a conserved caveolin-1 binding motif, **WNNMTWMQW**, in the ectodomain of HIV-1 transmembrane envelope glycoprotein gp41. We designed the synthetic CBD1 peptide SLEQI**WNNMTWMQW**DK, corresponding to the consensus caveolin-1 binding domain (CBD) in gp41, and showed that it elicits in rabbits the production of antibodies that inhibit infection of primary $CD4^+$ T lymphocytes by various primary HIV-1 isolates. Although a conserved and highly homologous caveolin-1 binding motif is present in the transmembrane envelope glycoprotein of different HIV-2 isolates, anti-CBD1 immune sera do not inhibit HIV-2 infection. Here we show that anti-CBD1 antibodies are directed against the conserved caveolin-1 binding motif **WNNMTWMQW** in the CBD1 epitope. In spite of this, anti-CBD1 antibodies do not react with the CBD2 peptide SLTPD**WNNMTWQEW**ER, corresponding to the potential consensus caveolin-1 binding domain in HIV-2. The presence of a conserved proline residue upstream of the caveolin-1 binding motif in CBD2 might affect the presentation of this motif, and thus account for the lack of reactivity of the immune sera. Anti-CBD1 antibodies therefore appear to be directed against a conformational epitope mimicked by the synthetic CBD1 peptide. In accordance with this, anti-CBD1 immune sera react with the native but not denatured gp41. The reactivity of anti-CBD1 immune sera with a highly conserved conformational epitope could explain the broad inhibitory activity of such antipeptide antibodies against HIV-1 isolates of various clades.

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Introduction

The current B-cell epitope vaccine candidates have failed to generate broadly neutralizing antibodies against HIV-1 (McMichael & Hanke 2003) because they induce antibodies that are viral-isolate specific due to the sequence variability of HIV and thus have limited usefulness (Moore et al 2001). We recently reported that the CBD1 peptide corresponding to the conserved caveolin-1 binding domain (CBD) in the transmembrane envelope glycoprotein of HIV-1, gp41, elicits in rabbits the production of peptide-specific antibodies that have the capacity to inhibit infection of $CD4^+$ T lymphocytes by various HIV-1 isolates (Hovanessian et al 2004). Because of their capacity to elicit antibodies that inhibit the different clades of HIV-1, CBD1-based peptides represent the first synthetic universal B-cell epitope vaccine candidate for HIV/AIDS. Moreover, such peptides could also have an application as a therapeutic vaccine since CBD1-specific antibodies are rare in HIV-infected individuals.

A number of pathogens appear to use lipid rafts and caveolae as the endocytosis route to exert their pathogenic effects (Campbell et al 2001). Lipid rafts are glycolipid-enriched membrane microdomains that are fundamental in the lateral organization of the plasma membrane by forming mobile platforms, which are implicated in clustering of membrane proteins, endocytosis, signal transduction and membrane trafficking (Harder et al 1998; Brown & London 2000). In the case of HIV-1, several groups

have reported the implication of lipid rafts in viral entry (Manes et al 2000; Nisole et al 2002) and budding process (Nguyen & Hildreth 2000; Ono & Freed 2001). Consistent with this, the two major lipid raft constituents, cholesterol and sphingomyelin, promote gp41 aggregation at the surface membrane (Saez-Cirion et al 2002). Furthermore, depletion of cellular cholesterol by the drug methyl β-cyclodextrin renders primary cells and cell lines highly resistant to HIV-1-mediated syncytium formation and to infection by both X4 and R5 HIV-1 strains (Liao et al 2001). Lipid rafts have also been shown to play a critical role in the HIV-1 assembly and release that take place at the plasma membrane. Both HIV-1 Gag and envelope protein appear to be associated with lipid rafts during the budding of virus particles (Nguyen & Hildreth 2000; Ono & Freed 2001). It is now well demonstrated that HIV-1 buds from lipid raft domains where almost all steady-state Gag/GagPol and about 30% of Gag/Gag complexes are associated with detergent-resistant membrane (Halwani et al 2003). Finally, transcytosis of HIV across epithelial cells was shown to be mediated by the capacity of virus particles to bind glycosphingolipid galactosyl ceramide receptors in caveolae (Alfsen et al 2001).

During studies on HIV-1 entry into permissive cells (Nisole et al 2002), we observed that gp41 gets associated with detergent-insoluble complexes corresponding to lipid rafts that also contain caveolin-1, a scaffolding protein that organizes and concentrates specific ligands within the caveolae membrane (Smart et al 1999; Liu et al 2002). We identified a conventional but distinct caveolin-1 binding motif in the ectodomain of gp41, suggesting that it could be responsible for interaction with caveolin-1 (Hovanessian et al 2004). We designed the synthetic peptide CBD1 corresponding to the consensus domain in gp41 that contains such a motif. We first showed that this peptide binds specifically caveolin-1, thus demonstrating its implication for interaction of gp41 with caveolin-1. Indeed, under physiological conditions gp41 binds caveolin-1 and thus could be functional in virus infection. We then demonstrated that several CBD1-like peptides reproducibly elicit in rabbits the production of antibodies that inhibit infection of primary $CD4^+$ T lymphocytes by various primary HIV-1 isolates (clades A to G). The neutralizing antibodies from the immune sera could efficiently be purified by an affinity matrix constructed with the CBD1 peptide (Hovanessian et al 2004). The inhibition of HIV-1 infection by the rabbit anti-CBD1 sera is therefore due to the action of CBD1-specific antibodies. Viral particles preincubated with the immune sera loose their infectivity, while addition of the immune sera to virus-producing cultures results in the production of defective virus particles (Hovanessian et al 2004). The caveolin-binding domain therefore is exposed on HIV particles and also on virus-infected cells.

Intriguingly, the anti-CBD1 immune sera that neutralize HIV-1 infection have very little or no effect on HIV-2 infection (Hovanessian et al 2004), in spite of the fact that the transmembrane envelope glycoprotein of HIV-2 also contains a conserved caveolin-1 binding motif homologous to that in the CBD1 epitope. Here we show that anti-CBD1 immune sera, which in an ELISA test strongly react with several peptides containing caveolin-1 binding motif, do not cross-react with the CBD2 peptide corresponding to the potential caveolin-1 binding domain in HIV-2. The lack of cross-reactivity of anti-CBD1 immune sera with the CBD2 peptide, and lack of neutralization of HIV-2 isolates, could be due to a specific conformation of the CBD2 peptide and the CBD2 epitope, respectively. This latter possibility might be triggered by the presence of a proline residue just preceding the conserved caveolin-1 binding motif in the CBD2 peptide/epitope.

Materials and Methods

Peptides

All peptides were synthesized in Fmoc chemistry by stepwise solid-phase methodology using a multichannel peptide synthesizer as previously described (Neimark & Briand 1993). Peptides were purified by reversed-phase HPLC and were identified by matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) spectra on a protein TOFTM mass spectrometer (Bruker, Wissembourg, France). For the biotinyl-CBD1, biotinyl-lysine was added at the alpha NH_2 terminus of the peptide. This lysine residue is naturally found at position 617 in gp41. On the other hand, the cysteine residue added at the N-terminal end of the cysteinyl-CBD1 peptide is not in the sequence of gp41. Overlapping peptides from the sequence of the HIV-1 envelope glycoprotein were obtained from the AIDS Research and Reference Program/NIAID, NIH (AIDS/NIH). The HIV-1 gp41 recombinant fragment 586–682 produced in Pichia pastoris was purchased from Viral Therapeutics, Inc. The recombinant HIV-1 gp120 produced in insect cells were obtained from the AIDS Research and Reference Program/NIAD, NIH.

Immunization of rabbits and ELISA

Rabbits (Fauves de Bourgogne) were injected at about 2-week intervals five times with the cysteinyl-CBD1 peptide (150 μg) using complete Freund's adjuvant (CFA). Rabbits KUR and LEO were immunized intradermaly for the first injection and intramuscularly for the following four injections, rabbits DIE and CAE were immunized intramuscularly, whereas rabbits NIA and MAR were immunized intradermally. The immune sera were titrated by enzyme-linked immunosorbent assay (ELISA) using 96-well plates (Maxisorp, Dynatech) coated with 2 μM of the CBD1 peptide or the overlapping gp41 peptides and incubated for 60 min at 37°C. The immune rabbit sera were added in serial dilutions and incubated for 60 min at 37°C. After washing, a mouse anti-rabbit immunoglobulin (IgG or IgM) conjugated with horseradish peroxidase was added. Following incubation and washing, o-phenylenediamine dihydrochloride substrate was added to the wells as described (Phan Chan Du et al 2002). Following colour development the reaction was quantitated at 450 nm. The antibody titres correspond to the reciprocal dilution of the respective rabbit serum, giving an OD value equal to 0.1 (measured at 450 nm).

Confocal microscopy

HeLa cells incubated with biotinyl-CBD1 were fixed with paraformaldehyde/Triton X-100 solution (Hovanessian et al 2000) and entry monitored using rabbit antibiotin antibodies (Enzo Diagnostics) and TR-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). At 20°C and below, CBD1 remains at the plasma membrane but at 37°C it enters cells, indicating that entry of CBD1 is by an active process.

Assay of HIV infection

$CD4^+$ T lymphocytes were propagated in RPMI-1640 containing 10% heat-inactivated fetal-calf serum and 50 IU mL^{-1} penicillin–streptomycin. $CD4^+$ lymphocytes were prepared from peripheral blood mononuclear cells after depletion of $CD8^+$ cells using Dynabeads M-450 CD8. After 3 days' stimulation with phytohemagglutinin, cells were suspended in fresh culture medium containing IL-2 and infected after 24 h (Hovanessian et al 2004). The maximum peak of virus production in such primary $CD4^+$ cells occurred at 4–6 days post-infection. The inhibitory activity of anti-CBD1 immune sera against HIV infection was assayed by preincubation (20°C, 20 min) of a given HIV isolate with various dilutions of the immune serum before incubation (37°C, 90 min) with permissive cells. The primary HIV-1 isolate used here was HIV-1 BZ167.

Statistical analysis

Levels of statistical significance were assessed using Student's *t*-test. Significant differences were judged for $P < 0.05$.

Results and Discussion

The distinct features of the CBD1 epitope

Caveolin-1 has a scaffolding domain located in the juxtamembranous region of the N-terminal domain, referred to as the caveolin-scaffolding domain, which is responsible for the oligomerization of caveolin-1 and represents the site for binding to various proteins (Okamoto et al 1998). By using the caveolin-scaffolding domain as a receptor, two binding motifs, ΦXΦXXXXΦ and ΦXXXXΦXXΦ, have been isolated from a phage display library, where Φ is any one of the aromatic amino acid residues W, F or Y, whereas X is generally a non-aromatic amino acid residue (Couet et al 1997). Interestingly, one of these two motifs is found in most of the caveolin-1 binding proteins (Liu et al 2002). A conventional caveolin-binding motif of the type ΦXXXXΦXXΦ is found in the ectodomain of the transmembrane envelope glycoprotein (Figure 1) of each one of the HIV-1, HIV-2 and SIV isolates with the three aromatic amino acid (Φ) residues as tryptophan (Hovanessian et al 2004). The synthetic CBD1 peptide corresponding to the consensus caveolin-1 binding domain in HIV-1 has the capacity to bind the caveolin-1 found in crude cell extracts in a specific dose-dependent manner. On intact cells, the CBD1 peptide causes clustering of caveolin-1 in the plasma membrane, resulting in the colocalization of the CBD1 peptide with caveolin-1, as revealed by laser-scanning confocal-immunofluorescence microscopy (Hovanessian et al 2004). Following binding, the CBD1 peptide enters cells by an active process since entry occurs at 37°C (Figure 2) but not at 20°C (not shown).

The CBD1 peptide composed of 16 amino acids is located at amino acid position 618 to 633: SLEQI**W**NNMT**W**MQ **W**DK (Hovanessian et al 2004). The distinct feature of the

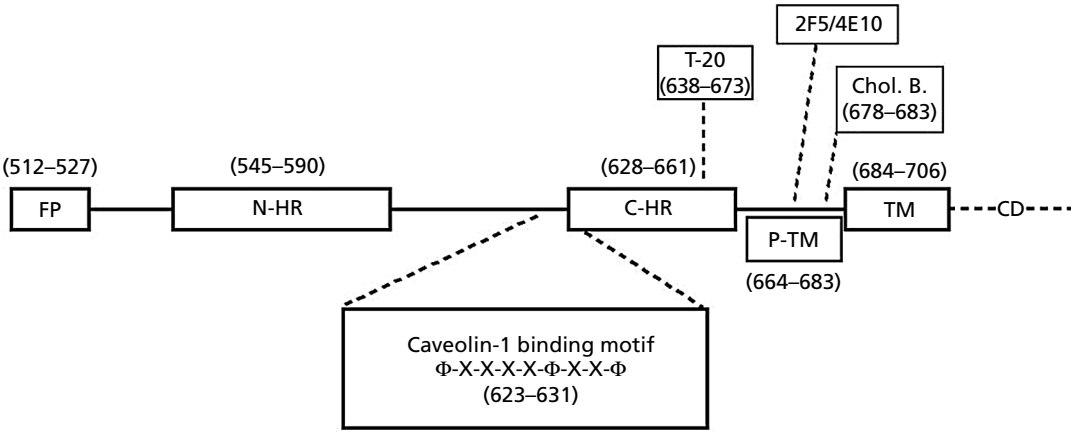

Figure 1 A schematic drawing of HIV-1 gp41 showing the location of the conserved caveolin-1 binding motif (ΦXXXXΦXXΦ). The important functional domains of gp41 ectodomain are the fusion peptide (FP) that becomes inserted into the membrane of HIV-target cells, the transmembrane region (TM) responsible for anchorage in the plasma membrane, the two hydrophobic heptad repeat domains characteristic of coiled-coils (N-HR and C-HR) (Eckert & Kim 2001); the conserved tryptophan-rich domain adjacent to TM (P-TM) that appears to cooperate with FP (Salzwedel et al 1999; Suarez et al 2000; Saez-Cirion et al 2002). The last five amino acids of P-TM (LWYIK) bind cholesterol (Vincent et al 2002). The P-TM also contains the core representative ELDKWA and NWFDIT epitope of the two broadly neutralizing human monoclonal antibodies (2F5 and mAb 4E10, respectively) (Cardoso et al 2005; Ofek et al 2004). The synthetic peptide T-20 is used in clinics as an inhibitor of the gp41-mediated fusion process (Kilby et al 1998). CD, cytoplasmic domain.

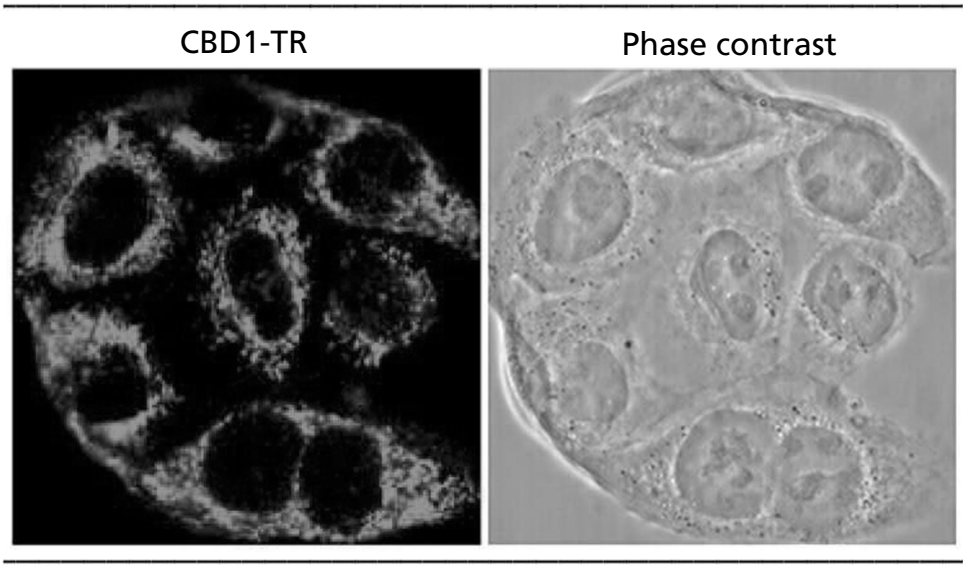

Figure 2 Entry of CBD1 into cells. HeLa cells were incubated with the biotinyl-CBD1 peptide (5 μM) for 45 min at 37°C before washing and fixation with paraformaldehyde/Triton X-100 solution. Entry of CBD1 was monitored using rabbit antibiotin antibodies and Texas red-conjugated donkey anti-rabbit IgG. A scan of a cross-section towards the middle of the cell monolayer showing the CBD1 and the corresponding phase contrast are presented. The experimental procedure for the preparation of slides and immunofluorescence confocal microscopy were as described previously (Hovanessian et al 2000).

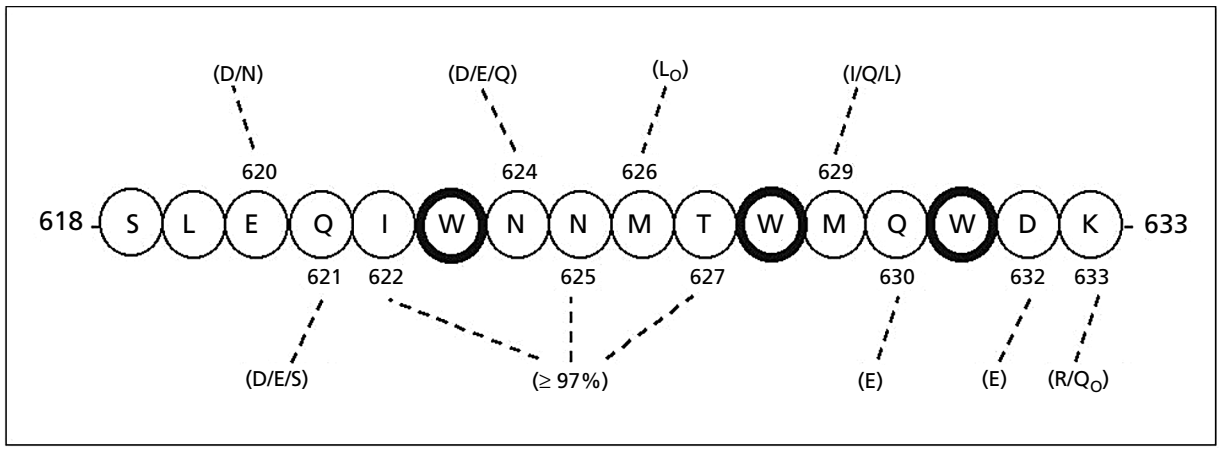

Figure 3 The conservation of the caveolin-1 binding domain in the ectodomain of gp41. The sequence of the CBD1 peptide corresponds to amino acids Ser-Leu-Glu-Gln-Ile-Trp-Asn-Asn-Met-Thr-Trp-Met-Gln-Trp-Asp-Lys. CBD1 is located at amino acid residues 618–633 in the consensus sequence in the gp41 ectodomain. The three tryptophan residues (W in bold) in the caveolin-binding motif are conserved among more than 850 isolates of HIV-1. The percentage conservation is given in brackets for some amino acid residues while for others the most frequent amino acid is given in brackets. The relatively conserved M^{626} residue is L in HIV-1 type O. The numbering of the CBD1 peptide is according to the consensus amino acid sequence of the gp41 ectodomain (Dong et al 2001).

caveolin-1 binding motif ^{623}**W**NNMT**W**MQ**W**^{631} in the CBD1 peptide is the three conserved tryptophan residues (Figure 3). The X-ray crystal structure of gp41 predicts that W^{628} and W^{631} are involved in the binding of C-HR domain to the small pocket in the hydrophobic groove of the N-HR trimeric coiled coil (Chan et al 1998), thus emphasizing the importance of conserving these aromatic residues as tryptophan. Point mutation of W^{628} to either A or F has been reported to impair the cleavage of the envelope precursor gp160 (Weng et al 2000). In addition to the three W residues, I^{622} and T^{627} are also conserved residues in the caveolin-1 binding domain. The mutation of I^{622} into A has no apparent effect on the synthesis of the envelope glycoprotein precursor gp160, but the generation of mature gp41 is completely prevented (Jacobs et al 2005), suggesting that this residue is necessary for proper processing and/or cleavage of gp160. The ^{625}NMT^{627} sequence is highly conserved, most probably because of the presence of an N-linked glycosylation site (N-X-T), which is one of the N-linked glycosylation sites in the gp41 ectodomain (Johnson et al 2001). The other residues

in the CBD1 peptide, $S^{618}/M^{629}/Q^{630}/D^{632}$, are 90–97% conserved, whereas $E^{620}/Q^{621}/N^{624}/K^{633}$ are slightly variable but their variation is mostly semi-conservative (Kuiken et al 2000; Dong et al 2001). The strong conservation of the individual amino acid residues of the CBD1 epitope, as well as gp41 binding to caveolin-1, suggests that there is a constant selective pressure to preserve this sequence for a specific function in the HIV infectious cycle. Moreover, there could be additional selective pressure because of the structural requirements necessary for processing of the envelope glycoprotein precursor.

Comparison of the **^{623}WNNMTWMQW^{631}** sequence in gp41 with amino acid sequences deduced from eukaryotic and non-eukaryotic cDNA banks by Blast FAQs analysis does not reveal the existence of any homologous sequence. It is therefore very unlikely that the CBD1-based peptides elicit any cross-reactive immune responses against caveolin-1 binding proteins that contain the ΦXXXXΦXXΦ type motif.

Fine epitope mapping of the rabbit immune sera against the CBD1 peptide

As we reported previously (Hovanessian et al 2004), rabbits injected with the cysteinyl CBD1 peptide elicit the production of antibodies that inhibit HIV-1 infection. For example, rabbit NIA and MAR immune serum at 100-fold dilution exerted 81 and 84% inhibition of HIV-1 infection. Assay of the neutralizing activity of anti-CBD1 immune sera was carried out using primary $CD4^+$ T lymphocytes infected by the primary HIV-1 BZ167 isolate (Materials and Methods).

In order to characterize the epitope of anti-CBD1 antibodies, these immune neutralizing sera were titrated by ELISA against the CBD1 peptide as well as five peptides (6362, 6363, 6364, 6365 and 6366) containing various segments of the caveolin-1 binding domain in HIV-1 MN gp41. In these experiments we also included a shorter version of the CBD1 peptide, referred to as the CBD1/M peptide, which lacks the first four amino acid residues, i.e. represents mainly the caveolin-1 binding motif in the CBD1 epitope (Table 1). The reactivity of these immune sera with various peptides appeared to require the presence of the complete caveolin-1 binding motif. Indeed, no reactivity occurred with peptides 6362, 6363 and 6366 in which the caveolin-1 binding motif is truncated at the N- or C-terminal end. On the other hand, significant cross-reactivity occurred with peptides 6364 and 6365, which expressed nine out of nine and eight out of nine residues of the caveolin-1 binding motif ^{623}**WNNMTWMQW**^{631}, respectively. The higher reactivity with the 6364 peptide might be accounted for in part by the presence of the conserved I^{622} adjacent to the motif. Overall, these data indicate that the immune sera can react with the caveolin-1 binding motif, although the N- and C-terminal amino acid residues flanking this motif are different from the corresponding residues in the CBD1 peptide. Thus antibodies elicited against the CBD1 peptide are directed against the highly conserved caveolin-1 binding motif. In accordance with this, the immune sera react strongly with the CBD1/M peptide that presents all the residues of the caveolin-1 binding motif. The reactivity of antibodies is reduced or completely abolished with peptides in which the motif is partially truncated at the N- or C-terminal end (Table 1).

The reactivity of anti-CBD1 immune sera with the conserved caveolin-1 binding motif could account for its capacity to neutralize a wide spectrum of HIV-1 isolates by binding to the caveolin-1 binding motif in gp41. Consistent with this, the anti-CBD1 immune sera NIA and MAR react in ELISA with a recombinant preparation of gp41. The specificity of this interaction is demonstrated by the lack of reactivity of the immune sera with a recombinant preparation of the HIV-1 external envelope glycoprotein gp120 (Table 1). Interestingly, the reactivity of the immune sera with gp41 is completely abolished when gp41 is denatured by sodium dodecyl sulfate (assayed by SDS-PAGE immunoblotting experiments;

Table 1 The major target of anti-CBD1 antibodies is the caveolin-binding motif

Peptide	Amino acid sequence	NIA	MAR
CBD1	SLEQI**W**NNMT**W**MQ**W**DK	1.83 ± 0.37	1.56 ± 0.25
6362	ASWSNKSLDDI**W**NNM	< 0.02	< 0.02
6363	NKSLDDI**W**NNMT**W**MQ	< 0.02	< 0.02
6364	DDI**W**NNMT**W**MQ**W**ERE	0.47 ± 0.06	0.38 ± 0.06
6365	NNMT**W**MQ**W**EREIDNY	0.21 ± 0.04	0.18 ± 0.03
6366	**W**MQ**W**EREIDNYTSLI	< 0.02	< 0.02
CBD1/M	I**W**NNMT**W**MQ**W**DK	1.75 ± 0.14	1.61 ± 0.18
gp41	(Recombinant)	0.39 ± 0.07	0.34 ± 0.08
gp120	(Recombinant)	< 0.02	< 0.02

The immune sera collected 2 weeks after the fifth immunization of rabbits NIA and MAR with the cysteinyl-CBD1 peptide were titrated by ELISA against the CBD1, CBD1/M and overlapping peptides 6362, 6363, 6364, 6365 and 6366 containing various segments of the CBD in the HIV-1 MN gp41. The immune sera were also titrated against a recombinant preparation of the HIV-1 external envelope glycoprotein gp120 and gp41 (Materials and Methods). The OD values $\pm$ s.d. correspond to the mean of triplicate samples at 1/500 dilution of the respective immune serum. The tryptophan residues in the caveolin-binding motif are in bold letters. The CBD1/M peptide represents the conserved caveolin-1 binding motif core in gp41.

not shown). Consequently, the reactivity of anti-CBD1 antibodies with gp41 appears to require the conformational integrity of the CBD1 epitope.

Immune sera against the CBD1 peptide do not cross-react with the CBD2 peptide

We have previously shown that anti-CBD1 immune sera inhibits infection of $CD4^+$ T lymphocytes by various HIV-1 isolates but has no effect on HIV-2 isolates, in spite of the existence of a conserved caveolin-1 binding motif in the transmembrane envelope glycoprotein of HIV-2 (Hovanessian et al 2004). Consequently, the marked inhibition of HIV-1 isolates by the immune sera should be due to a specific event exerted by anti-CBD1 antibodies on the caveolin-1 binding motif in HIV-1 gp41. Consistent with this, the anti-CBD1 immune sera do not cross-react with the CBD2 peptide corresponding to the potential caveolin-1 binding domain deduced from the consensus sequence of the transmembrane envelope glycoprotein of HIV-2. Figure 4 and Table 2 give the results of ELISA tests showing that while several anti-CBD1 immune sera react strongly with the CBD1 peptide and the caveolin-1 binding motif core (peptide CBD1/M), they manifest no apparent reactivity with the CBD2 peptide. This might be due to structural and/or conformational differences of the caveolin-1 binding motif in the CBD2 peptides. Indeed, although the caveolin binding motifs ^{623}WNNMTWMQW^{631} and ^{629}WNNMTWQEW^{637} (amino acid numbering as in reference (Rey-Cuillé et al 1994)) in the CBD1 and CBD2, respectively, are homologous, the two amino acid residues just preceding the caveolin binding motif in CBD2 are different from the corresponding residues in CBD1. Firstly, I^{622} that is conserved in HIV-1 is replaced either by D, E, N, or R in HIV-2. Secondly, the Q^{621} that is semi-conserved in HIV-1 is replaced by a proline (P) residue in HIV-2, which is highly conserved among HIV-2 and SIV isolates. The presence of a proline residue in a peptide could induce the formation of a beta-turn and thus might have a dramatic effect on the conformation of the peptide. Moreover, the interaction of a proline residue with an aromatic residue could stabilize turn structures (Leitgeb & Toth 2005). The fact that anti-CBD1 immune sera could still react with the CBD1-related peptide 6365 lacking I^{622} (NNMT**W**MQ**W**EREIDNY, Table 1) indicates that the absence of this isoleucine residue is less critical than the presence of the proline residue in the CBD2 epitope. Thus, it is very likely that a structural modification induced by the conserved proline residue in the CBD2 epitope is responsible for the lack of reactivity of anti-CBD1 antibodies with the caveolin-1 binding motif presented by the CBD2 peptide. Overall these data are consistent with the conclusion that the CBD1 peptide elicits antibodies that recognize a conformational epitope provided by the CBD1 peptide, i.e. the conserved caveolin-1 binding motif. Nevertheless, we cannot rule out the possibility of the existence of a small proportion of anti-CBD1 antibodies that react with specific linear epitopes. Recently, a human IgM mAb C37 binding to the sequence IWNNM in the caveolin-binding motif was isolated from an HIV-seropositive long-term non-disease progressing patient (Cao et al 2004). Whether or not mAb C37 has the capacity to neutralize HIV-1 infection has not yet been reported.

The important sequence homology between the caveolin-1 binding motif in HIV-1 and HIV-2, the lack of reactivity of anti-CBD1 immune serum with the homologous caveolin-1 binding motif in the CBD2 peptide, and the lack of HIV-2 neutralization by anti-CBD1 antibodies favours the suggestion that such antibodies react with a specific conformational epitope in HIV-1 gp41. In accord with this, anti-CBD1 antibodies do not react with the denatured form of gp41. The reactivity of anti-CBD1 antibodies with a conserved conformational epitope is consistent with the broad neutralizing activity of such antibodies against various HIV-1 isolates (Hovanessian et al 2004).

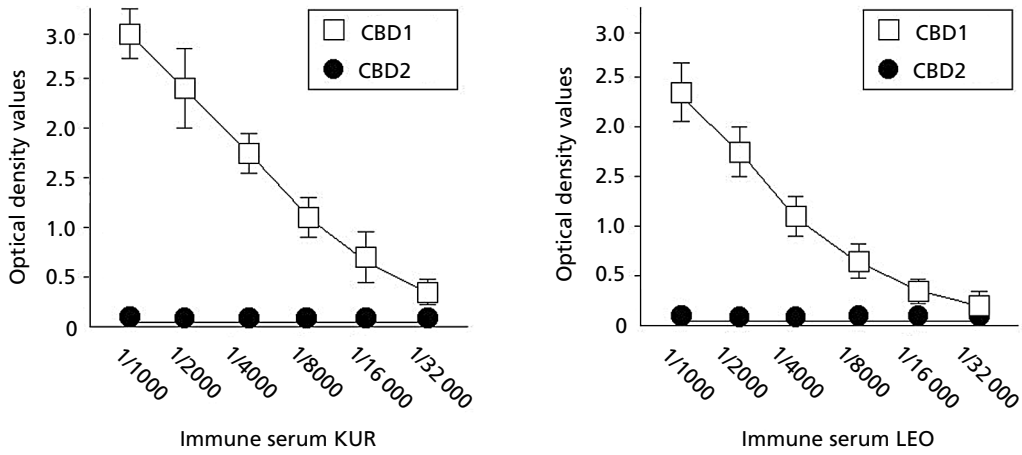

Figure 4 The lack of reactivity of the anti-CBD1 rabbit KUR and LEO immune serum with CBD2 peptide. Rabbit KUR and LEO were injected intramuscularly (IM) with the cysteinyl-CBD1 peptide using CFA as adjuvant, and the immune serum collected 2 weeks after the fifth injection was titrated by ELISA against the cysteinyl-CBD1 and -CBD2 peptides. The ordinate gives the OD values (at 450 nm) observed at 1000 to 32 000 dilutions (abscissa). See Table 2 for the amino acid sequence of the CBD1 and CBD2 peptide.

Table 2 Anti-CBD1 antibodies react with the caveolin-binding motif core but do not react with the homologous motif in the CBD2 peptide

CBD1: S-L-E-Q-I-**W**-N-N-M-T-**W**-M-Q-**W**-D-K
CBD2: S-L-T-P-D-**W**-N-N-M-T-**W**-Q-E-**W**-E-R

Rabbit serum	ELISA: CBD1	ELISA: CBD2	ELISA: CBD1/M*
Rabbit control	< 0.02	< 0.02	< 0.02
Rabbit KUR	2.49 ± 0.15	< 0.02	2.46 ± 0.05
Rabbit LEO	1.88 ± 0.11	< 0.02	1.65 ± 0.25
Rabbit DIE	1.89 ± 0.08	< 0.02	1.78 ± 0.18
Rabbit CAE	1.57 ± 0.13	< 0.02	1.68 ± 0.22
Rabbit NIA	2.27 ± 0.09	< 0.02	2.13 ± 0.16
Rabbit MAR	1.75 ± 0.15	< 0.02	1.81 ± 0.19

Immune sera collected 2 weeks after the fifth injection of rabbits (KUR, LEO, DIE, CAE, NIA, MAR) were titrated by ELISA against peptides CBD1, CBD1/M and CBD2. The OD values ± s.d. correspond to the mean of triplicate samples at 1/400 dilution of the respective immune serum. The tryptophan residues in the caveolin-binding motif are in bold letters.
*This peptide represents the conserved caveolin-1 binding motif core in gp41.

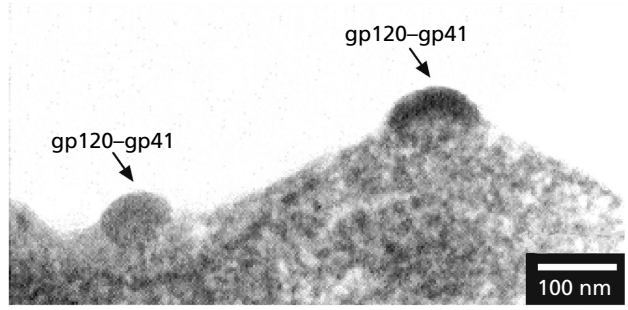

Figure 5 Electron micrograph showing condensed regions at the plasma membrane of CEM cells expressing functional HIV-1 gp120–gp41 complex. Human $CD4^+$ lymphoblastoid CEM cells were infected by the vaccinia virus recombinant expressing HIV-1 LAI *env* gene (VVTG 9.1) and 48 h post-infection cells were fixed in 2.5% glutaraldehyde and processed for the preparation of electron microscopy grids (Laurent-Crawford et al 1993). This electron micrograph represents an ultra-thin section stained with uranyl acetate and lead citrate. It should be noted that the gp120–gp41 complex expressed at the plasma membrane of such CEM cells is functional since it triggers apoptosis and membrane fusion following interaction with $CD4^+$ bystander cells (Laurent-Crawford et al 1993).

The mechanism of action of anti-CBD1 antibodies against HIV-1 infection

The CBD1 epitope located at the end of the loop between the helical N-HR and C-HR regions and overlapping several residues of the C-HR should probably be exposed both on HIV-1 particles and HIV-1-producing cells since anti-CBD1 antibodies have the capacity to neutralize free virus and cause aggregation of gp41 in the plasma membrane of HIV-infected cells, resulting in the production of defective virus particles with reduced capacity to infect permissive cells (Hovanessian et al 2004). Indeed, as a consequence of gp41 aggregation in the plasma membrane, the incorporation of gp41 in the budding virus becomes reduced, leading to decreased levels of virus-associated gp120. These observations pointed out that caveolin-1 might also be implicated in the HIV budding process during the assembly of viral components at lipid raft domains in the plasma membrane of HIV-1-producing cells (Campbell et al 2001; Ono & Freed 2001). The interaction of the newly generated gp41 with caveolin-1 in the plasma membrane might trigger caveolin oligomerization, which in turn could cause clustering of the gp120–gp41 complex in the plasma membrane. Indeed, while lipid rafts appear to be small in size and dispersed all around the plasma membrane of non-polarized cells, the interaction of raft-associated proteins with their ligands or their cross-linking with antibodies leads to the oligomerization of raft components (Harder et al 1998). Electron microscopy studies have shown that the distribution of gp120–gp41 complex in HIV envelope glycoprotein-expressing cells is restricted to electron-dense crescent structures at the plasma membrane (Kadsumoto et al 1990). Similarly, electron micrographs from expressing functional HIV-1 envelope glycoproteins (gp120–gp41 complex) show condensed regions at the plasma membrane, probably as a result of the clustering of the gp120–gp41 complexes (Figure 5 and Laurent-Crawford et al 1993). This clustering of the gp120–gp41 complex at the plasma membrane could be essential for the proper incorporation of the functional envelope glycoprotein complex in the membrane of the budding virus.

The capacity of anti-CBD1 antibodies to react with gp41 expressed on the surface of HIV-1-producing cells suggests that in vivo anti-CBD1 antibodies might also trigger elimination of HIV-infected cells by Fc-mediated effector systems, such as antibody-dependent cellular and complement-mediated cytotoxicity (Parren & Burton 2001). Moreover, defective HIV particles produced in the presence of anti-CBD1 antibodies could be engaged by the host immune system for the generation of humoral and cellular immune responses against other viral proteins, as has been reported recently in response to HIV pseudovirion particles (Chen et al 2005).

Conclusion

The main target of anti-CBD1 antibodies is the conserved caveolin-1 binding motif in the CBD1 peptide. In spite of this, such antibodies manifest no apparent reactivity with the homologous caveolin-1 binding motif in the CBD2 peptide, corresponding to the transmembrane envelope glycoprotein of HIV-2. Here we suggest that the lack of cross-reactivity with the caveolin-1 binding motif in the CBD2 peptide might be due to a modified conformation of this motif when presented in the context of the CBD2 peptide. Consequently, because of its conformation, the CBD1 peptide elicits antibodies that have the capacity to react with a conformational epitope that is specific to the caveolin-1 binding domain in native gp41 of HIV-1.

Consistent with this, anti-CBD1 antibodies exert a broad inhibitory activity against HIV-1 isolates of various clades, but have no inhibitory effect on HIV-2 isolates (Hovanessian et al 2004). CBD1-based peptides therefore provide a unique B-cell epitope vaccine for the development of effective prophylactic vaccines for use against HIV-1 infection. Such vaccines could be used in association with CTL-based vaccines, which are currently in clinical trials.

The CBD1 peptide vaccine provides several advantages over conventional vaccines in that highly purified peptides may be made in larger quantities and at lower cost. In addition, its simple antigenic composition could offer protection with fewer side-effects. Furthermore, the CBD1 vaccine should elicit broadly neutralizing antibodies because of its conserved nature among various HIV-1 isolates. Finally, because of the capacity of these antibodies to act also on infected cells, and since natural antibodies against the CBD1-epitope are at low titre and present in only a small proportion of HIV-infected individuals, the CBD1 vaccine could also be used as a therapeutic vaccine in HIV-infected individuals.

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